18. ENDOCRINE DISRUPTORS IN SEWAGE TREATMENT PLANTS

Summary of three-year nationwide survey and recent research at PWRI

Presenter

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Abstract

The field study on suspected endocrine disruptors (EDs) was conducted at 47 municipal sewage treatment plants (STPs) in Japan. Either composite or grab samples were collected at the STPs, and analyzed for EDs and related substances. Estrogen-like activities (estrogenicities) of sewage and treated sewage were determined using a DNA recombinant yeast strain containing build-in estrogen receptors. The suspected EDs commonly found in influent sewage and effluent of the secondary treatment process include nonylphenol, bisphenol, 2,4-dichlorophenol, 2-ethylhexyl phthalate, di-2-ethylhexyl adipate, benzophenone, 17β–estradiol, and nonylphenol ethoxylates. These substances in influent sewage were removed to some extent in the primary and secondary treatment processes. Farther reduction of the concerned substances was observed in the advanced treatment processes including sand filtration, ozonation, activated carbon adsorption, and membrane filtration/reverse osmosis (MF/RO) filtration. The chemicals of concern (COC), suspected EDs and related substances, remaining in sludge produced in the STPs appear to be decomposed in an incineration process, as their concentrations in ash were below their detection limits. The results of the yeast assays indicate that the estrogenicity of sewage can be reduced effectively in the municipal STPs, and that approximately one quarter of the observed estrogenicity is associated with 17β–estradiol and estrone present in the influent sewage and with estrone in the final discharge. The COC concentrations and their estrogenic potential suggest that estrone, 17β–estradiol and nonylphenol are more important compounds among the COC studied in the nation-wide survey. In many of the STPs studied, however, there are some differences between the estrogenicity determined using the yeast assays and the estrogenic effects estimated from the concentrations of COC and their estrogenic potentials. These differences and results from our fractionation study imply presence of unidentified estrogen-like substances in the influent sewage as well as in the treated sewage. Male-carp breeding experiments were conducted by PWRI with secondary effluent from a sewage treatment plant. Results from these experiments indicate induction of vitellogenin in their blood, but this induction was not observed regularly, suggesting that this phenomenon might be dependent upon the balance between the extents of endogenous and exogenous hormone activity.

Key words: endocrine disruptors, estrogenicity, 17β–estradiol, estrone, sewage treatment plants

1. Introduction

Rivers and streams carry domestic wastewaters and a number of substances released as products of various human activities in the river basins. In recent years, a new problem has emerged in our water environment, namely endocrine disruptors (EDs) that may adversely affect the reproductive functions of human beings and wild life. The UK Environment Agency conducted research on the effects of EDs on fish in many English rivers, and addressed their potential problems in their rivers[1]. Immediately after the UKEA[1] reported their findings, the Ministry of Land, Infrastructure and Transport (MLIT, previously the Ministry of Construction) of Japan acted by classifying the EDs problem as an emergency issue of the environment that requires immediate actions to deal with the problem. Since there were very few studies on the impacts of EDs in the water environments, MLIT determined that real situations of

Japanese rivers and sewerages must be immediately evaluated and that a nationwide fact-finding study must be conducted jointly with the Japan Environmental Agency (JEA).

The estrogenic effects by the discharges from sewage treatment plants (STPs) are of great concern because feminization of male fish downstream of STPs has been reported in England and the United States.[1,2,3,4] The discharges from industries[5] and agricultural activities[6] have been reported to cause feminization of male fish. As a first step, MLIT decided to focus on nationwide surveys on EDs that might be released into water bodies from domestic and industrial activities.[7] From FY1999 to FY2001, a sewerage study was conducted at 47 STPs, aiming at clarifying the present state of EDs in urban wastewaters. The urban wastewater streams have been thought to be one of the key routs for EDs to enter the water environment. In this paper, we first summarize the state of EDs including suspected EDs and related substances as chemicals of concern (COC), found in sewage. The initial findings reported herein are primarily based on the above-mentioned surveys conducted by MLIT. Then, we analyze the fate of COC in the STPs and the effectiveness of the STPs to remove such compounds. Based on results of the DNA recombinant yeast assay (the YES assay), we examine the estrogen-like activities of untreated sewage and sewage treated to a varying degree in the STP. Finally, we summarize our recent findings on: a) improvement of the ELISA method for 17β–estradiol in sewage; b) estimation of substances causing estrogen-like activity based on the fractionation method; and c) male-carp breeding experiments with secondary effluent of a STP.

2. Methods and Nationwide Survey

2.1 Sewage treatment plants studied

The three-year study was conducted in the cooperation with the city of Tokyo, five prefectures (Ibaraki, Saitama, Shiga, Kyoto, Osaka), and nine major cities (Sapporo, Sendai, Kawasaki, Yokohama, Nagoya, Kyoto, Osaka, Kobe, Fukuoka). In FY1998, the surveys were conducted at total 27 STPs. These surveys covered 10 STPs in summer, 20 STPs in fall, and 19 STPs in winter. The FY1999 surveys were performed at 38 STPs: 3 STPs were studied in spring, 35 STPs in summer, and 31 STPs in fall. In FY2000, the surveys were conducted at 20 STPs covering 10 STPs in summer and 20 STPs in fall. Total 47 STPs were studied over 3 years.

2.2 Chemicals of concern

In May 1998, Japan Environment Agency[8] released the document titled "The Strategic Programs on Environmental Endocrine Disruptors '98" (SPEED '98) that lists approximately 70 chemicals as the substances suspected to have endocrine disrupting effects. The SPEED '98 gives the following specific approaches: 1) Promotion of investigations and surveillance for the pollution with EDs, sources of EDs, and adverse effects of EDs on wildlife and human beings; 2) Promotion of development of research and testing methods; 3) Promotion of environmental risk assessment and management; and 4) Strengthening of international networks.

In the first year of this study, 25 chemicals were chosen as COC from the SPEED '98 list. The selected compounds are suspected EDs[8] and those that might be discharged in sewage because of possible usages in households and/or industries. The selected COC include polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), alkylphenols (4-t-butylphenol, 4-n-pentylphenol, 4-n-hexylphenol, 4-n-heptylphenol, 4-t-octylphenol, 4-n-octylphenol and nonylphenol), bisphenol A, 2,4-dichlorophenol, phthalates and adipate (diethyl phthalate, dipropyl phthalate, di-n-butyl phthalate, dipentyl phthalate, dihexyl phthalate, butyl benzyl phthalate, di-(2-ethylhexyl) phthalate, dicyclohexyl phthalate and dietylhexyl adipate), aromatic compounds (benzo(a)pyrene, benzophenone, 4-nitrotoluene, octachlorostyrene, styrene dimmer, styrene trimer), and VOC (n-butylbenzene). The second-year study focused on 11 chemicals, which were chosen because they exhibited high occurrences in the first year study and their concentrations in influent sewage exceeded their quantification limits (QLs). In the third year, 6 compounds were selected as COC, including nonylphenol, bisphenol A, and di-2-ethylhexyl phthalate, benzophenone, nonylphenol ethoxylates, and 17â-estradiol. These compounds were chosen because of their high occurrences and difficulty in removing from wastewaters.

In our study, in addition to the EDs selected in SPEED' 98, nonylphenol ethoxylates and 17â-estradiol were included because the former compounds have been reported to produce nonylphenol and the latter is one of female hormones derived from human and animals. Nonylphenoxy acetates, estrone, and ethynyl estradiol were also investigated based on the facts that nonylphenoxy acetates are suspected to produce nonylphenol, estrone is natural

estrogen, and ethynyl estradiol is contraceptive substance. The analytical methods and their QLs for COC in the sewerage studies are summarized in Table 1.

Table 1. Principal methods for the analysis of endocrine disrupting chemicals used in the sewerage study

Substance	Analysis Method Principles		Minimum Quantification Limit	
	Water Sample	Sludge	water (μg/L)	sludge (μg/kg-dry)
4-n-octylphenol 4-t-octylphenol 4-t-butylphenol 4-n-pentyphenol 4-n-hexylphenol 4-heptylphenol Nonylphenols	After extraction with dichloromethane, it is cleaned-up with a silica-gel column (dichloromethane elution), dewatered and concentrated, then measured with a GC/MS-SIM	After Soxlet extraction, it is added to water. Then, it is solid-phase extracted, dewatered, concentrated and cleaned-up with a silica-gel column. After quantified with hexane, it is concentrated and measured with GC/MS-SIM and IS methods.	0.3	0.5
Di-2-ethylhexyl phthalate	After sodium chloride is added to it to extract with hexane, it is cleaned-up with a flori-gel column, concentrated and dewatered with a nitrogen draft, then measured with a GC/MS-SIM using surrogate standards	After supersonic-extracted with acetonitrile, it is dewatered, concentrated, cleaned up with a GPA column. Then, it is concentrated and measured with GC/MS-SIM method using surrogate method.	0.6	5
Butyl benzyl phthalate BBP Di-n-butyl phthalate DBP Diethyl phthalate Dipropyl phthalate Dipenthyl phthalate Dihexyl phthalate Dicyclohexyl phthalate			0.6	1
Di (2-ethylhexyl) adipate Benzo(a)pyrene 4-nitrotoluene	After sodium chloride is added to it to extract the hexane, it is cleaned-up with a silica-gel column, concentrated and dewatered with a nitrogen draft, then measured with a GC/MS-SIM. In addition a surrogate standard was used.		0.03	
Benzophenone		After supersonic-extracted with acetone, it is extracted with hexane after addition of sodium chloride. Then, it is dewatered, concentrated, and cleaned up with a silica-gel column. Finally, it is concentrated and measured with GC/MS-SIM and IS methods.	0.03	0.5
Bisphenol A 2,4-dichlorophenol	After extracted with dichloromethane, dewatered and concentrated, it is converted to TMS and measured with a GC/MS-SIM	After supersonic-extracted with acetone, it is extracted with dichloromethane after addition of sodium chloride. Then, after dewatered and concentrated, cleaned up with a silca-gel column, it is TM -derivatived and measured with GC/MS-SIM and IS methods.	0.03	0.5
Styrene dimer and trimer 1,3-diphenylpropane,2,4-diphenyl-1-buthan,cis-1,2-diphenylsyclobuthan,trans-1,2-diphenylcyclobuthane,2,4,6-triphenyl-1-hexene,1a-phenyl-4a-(1'-phenylethyl)tetrarine,1a-phenyl-4e-(1'phenylethyl)tetrarine,1e-phenyl-4a-(1'-phenylethyl)tetrarine,1e-phenyl-4e-(1'-phenylethyl)tetrarine	After sodium chloride is added to extract it with hexane, it is cleaned-up with a silica-gel column, concentrated and dewatered with a nitrogen draft, then measured with a GC/MS-SIM	After heated-and cycled extraction with 1M KOH-methanol, it is extracted with hexane after addition of sodium chloride solution. It is dewatered, concentrated, cleaned up with a silica-gel column and concentrated. It is measured with GC/MS-SIM and IS methods.	0.03	0.5
n-butylbenzene	Headspace-GC/MS-SIM with Internal standard(IS)		0.3	
octachlorostyrene Poly Brome Biphenyls	After filtered with a glass fiber filter, residual is supersonically extracted with acetone and the extract is added to the filtrate. After extracted with hexane, it is dewatered and concentrated, then measured with a GC/MS-SIM with Internal Standard method.		0.09	

Nonylphenol ethoxylates (n=1~4)	After filtered with a glass fiber filter, residual is supersonically extracted with acetone and the extract is added to the filtrate. After solid-phase extraction, it is eluted and cleaned with a silica-gel column and concentrated, then measured with a HPLC.	After Soxlet extraction with methanol, it is added to water. Then, it is solid-phase extracted, dewatered, concentrated and cleaned up with a silica-gel column. Then, it is concentrated and measured with HPLC.	0.6	3
(n≧5)			0.6	1
Nonylphenoxyacetates nonylphenoxy acetate nonylphenol monoethoxy acetate nonylphenol diethoxy acetate	After filtered with a glass fiber filter, residual is supersonically extracted with methanol and the extract is added to the filtrate. After solid phase extraction, it is methyl-derivatived, and is measured with GC/MS-SIM and internal standard method.		1.5	
17 β -estradiol(ELISA)	After SPE, the extract is dissolved into DMSO without decomposition of estradiol conjugates and is measured with an enzyme-linked immunosorbent assay (ELISA) kit	After extracted with methanol-acetic acid buffer solution, it is concentrated and added to water. Then, it is solid-phase extracted, dried and dissolved with DMSO. Finally it is measured with ELISA method using a kit by Assay-Design Corp.	0.0006	0.05
Estrogens(LC/MS/MS method) Estrone 17 β -estradiol ethynyl estradiol	After filtered with a glass fiber filter, residual is supersonically extracted with methanol and the extract is added to the filtrate. After solid phase extraction, it is cleaned-up with a froli-gel column and a thin layer chromatogram. Then, it is measured with LC/MS/MS-SIM using internal standard method.		0.0015	

2.3 Sampling locations in sewage treatment plants

Most of the STPs studied employ a conventional activated sludge process, while some apply step aeration, oxidation ditch, as well as advanced treatment for nitrogen and/or phosphorus removal employing coagulant-added activated sludge, circulated nitrification-denitrification process, anaerobic-oxic (AO) process, or anaerobic-anoxic-oxic (A2O) process. The size of the STPs studied ranges from 2,000 to 1,027,000 m^3/day. Fig. 1 shows a typical STP and the locations where samples were collected. Grab samples were collected in the first year, while composite sampling was generally applied in the second and third year. The composite samples were collected every three hours for a 24 hour period or collected at every two hours during daytime. The samples were collected from the influent of STP, influent and effluent of the primary settling tank, effluent of the final settling tank, and final effluent discharge following disinfection. Some of the STPs apply physicochemical processes as tertiary treatment, including rapid filtration, ozonation, activated carbon absorption, membrane filtration using micro-filtration (MF), or reverse osmosis (RO) aiming at reclamation and reuse of treated wastewater. In the physicochemical processes, samples were collected from the influent and effluent of each unit process. Sludge generated in the primary and final settling tanks, thickened sludge, anaerobically digested sludge, dewatered sludge, and ash from incinerator, were also collected.

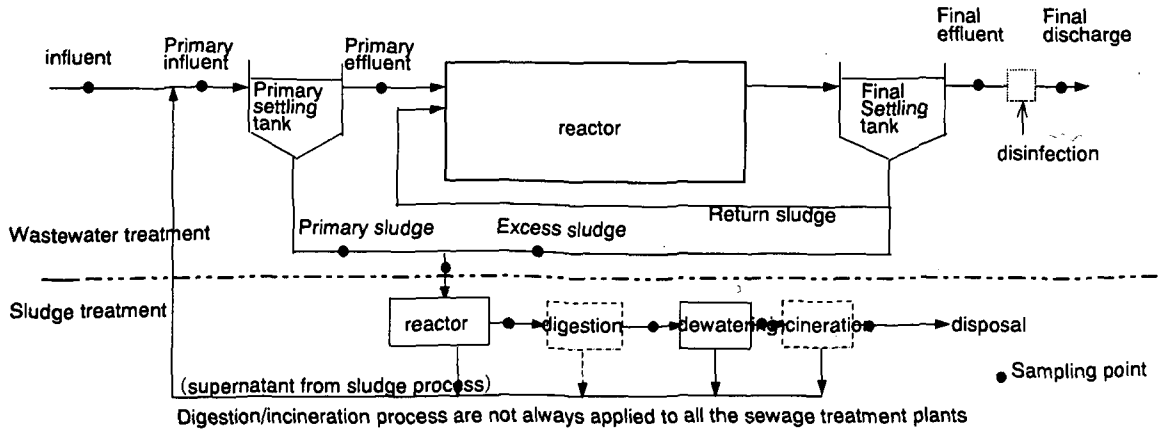


Fig. 1. Sampling points in Sewage Treatment Plants

2.4 Yeast estrogen screen assay

Substances that exhibit estrogenic activities in sewage are of significant concern. Although a number of such compounds are identified in the SPEED'98,[8] actual number of EDs present in the environment are unknown. At present, existing knowledge on EDs in a sewage treatment system is limited to develop a sound basis for planning to prevent their adverse effects. Therefore, efforts have been made to increase our understanding of these compounds. In this study, we evaluated the estrogen-like activities of untreated and treated sewage using DNA recombinant yeast provided by the courtesy of Prof. Sumpter, Brunel University in England.[9] In the DNA recombinant yeast, a human estrogen receptor gene is integrated into the yeast's genome in the nucleus and forms estrogen receptor elements. The estrogen receptor in the yeast is reactivated in binding with estrogen-like substances and the ligand-binding receptor unit expresses a reporter gene, *Lac-Z,* on the plasmid in the yeast. Then, β-galactosidase is produced due to the expression of *Lac-Z* and is secreted out of the yeast. The β-galactosidase produced under the series of reactions can change yellow color of a chromosomic agent, chlorophenol red-β-D-galactopyranside (CPRG), into red. Because production of β-galactosidase in the medium depends upon the amount of estrogen-like substances, measurement of absorbance by a spectrophotometer can estimate the amount of estrogen-like substances in the assay medium. This estrogen detection assay is named as the yeast estrogen-inducible expression system or YES,[1] and is applicable to sewage samples.[1,10,11,12,13] The YES assay protocol used in this study basically followed Yakou *et al.* [12] and Tanaka *et al.* [13] In brief, one liter of each sample was collected and pre-filtered with a glass fiber filter, GF/B, having approximately 1-ì m pores. The filtrate was extracted with a C18 solid phase extraction (SPE) cartridge (500 mg) that had been finished by conditioning and dewatered. Then the C18 SPE cartridge was eluted with 10 mL of methanol. The GF/B filter having suspended matters was also supersonic-extracted. Both the extracts were mixed and condensed into approximately 1 mL with a rotary evaporator. Then the methanol solution was purged by nitrogen gas and was dried. The residuals were dissolved in 100 ì L of DMSO, and used for the estrogen assay in dilution. The relative estrogenic strength of each sample was evaluated from EC50 estimated from its dose-response curve at the same absorbance of 17β-estradiol. The 17β-estradiol equivalent of the original sample can be estimated as the EC50 of 17β-estradiol divided by the EC50 of the test sample at a given concentration factor.

3. Results of chemical-based study

3.1 Concentrations in sewage treatment plants

3.1.1 Influent sewage concentration

For the 34 substances (suspected EDs and related substances) found in sewage in the influent of the STPs, the maximum concentration, 75th percentile, 50th percentile (median), minimum concentrations, and detection limit (DL) of each substance are presented in Fig. 2. Among the substances listed in SPEED '98, 10 were found below their DLs at all the STPs studied, while 15 substances were observed above their QLs in sewage in at least one of the STPs. These 15 substances are 4-*t*-butylphenol, 4-*n*-octylphenol, 4-*t*-octylphenol, nonylphenol, bisphenol A, 2,4-dichlorophenol, diethyl phthalate, di-*n*-butyl phthalate, di-2-ethylhexyl phthalate, butylbenzyl phthalate, benzo(a)pyrene, di-2-ethylhexyl adipate, benzophenone, stryrene dimers and trimers, and *n*-butyl benzene. Substances (EDs and related substances) that are not listed in SPEED '98 but were identified at levels greater than their QLs include nonylphenol ethoxlates, nonylphenol ethoxy acetates, 17β-estradiol, and estrone. Ethynyl estradiol was not found at any sewage treatment plants. Nonylphenol, di-ethyl phthalate, di-2-ethylhexyl phthalate and benzophenone,

nonylphenol ethoxylates, nonylphenol ethoxy acetate, 17 β -estradiol (measured by ELISA or LC/MS/MS method), and estrone were identified in all the STPs at levels greater than their QLs. Among alkylphenols, nonylphenol was identified most frequently and at the highest levels. Nonylphenol, nonylphenol ethoxylates, and nonylphenol ethoxy acetates existed on the order of 1 to 1000 ì g/L. Bisphenol A existed on the order of 0.1 to 10 ì g/L. Among the phthalate esters, di-2-ethylhexyl phthalate, diethyl phthalate, di-*n*-butyl phthalate, and butylbenzyl phthalate existed at relatively high concentrations. Among estrogens, 17 β -estradiol analyzed using the ELISA method was most frequently observed, and its concentration was one order of magnitude larger than the same substance analyzed using LC/MS/MS. This higher detection is likely due to cross-reactions of other substances that are similar to the structure of 17 β -estradiol in ELISA kit.[14] Estrone existed in sewage more prevalently than 17 β -estradiol on the basis of the data obtained by LC/MS/MS. As the concentrations of the estrogens range from few ng/L to 100 ng/L, the variation of the estrogens are relatively smaller than the other man-made substances used in industry or households. It is elucidated by many researchers[15] that nonylphenol ethoxylates are degraded to nonylphenol acetic acetate and to nonylphenol under anaerobic conditions. Assuming that the nonylphenol precursors have the potential to produce nonylphenol, nonylphenol and its related substances are expressed in terms of nonylphenol-equivalent unit (in mole unit). Based on the nonylphenol-equivalent unit (median values), the distribution of nonylphenol and its related substances is following: nonylphenol, 3%; nonylphenol ethoxylates, 33%; and nonylphenol ethoxy acetates, 64%. Note that nonylphenol contributes only small fraction (3%) of total nonylphenol-equivalent. Among estrogens that were measured by LC/MS/MS, the concentration of estrone was 4 times greater than that of 17 β -estradiol (based on their median values).

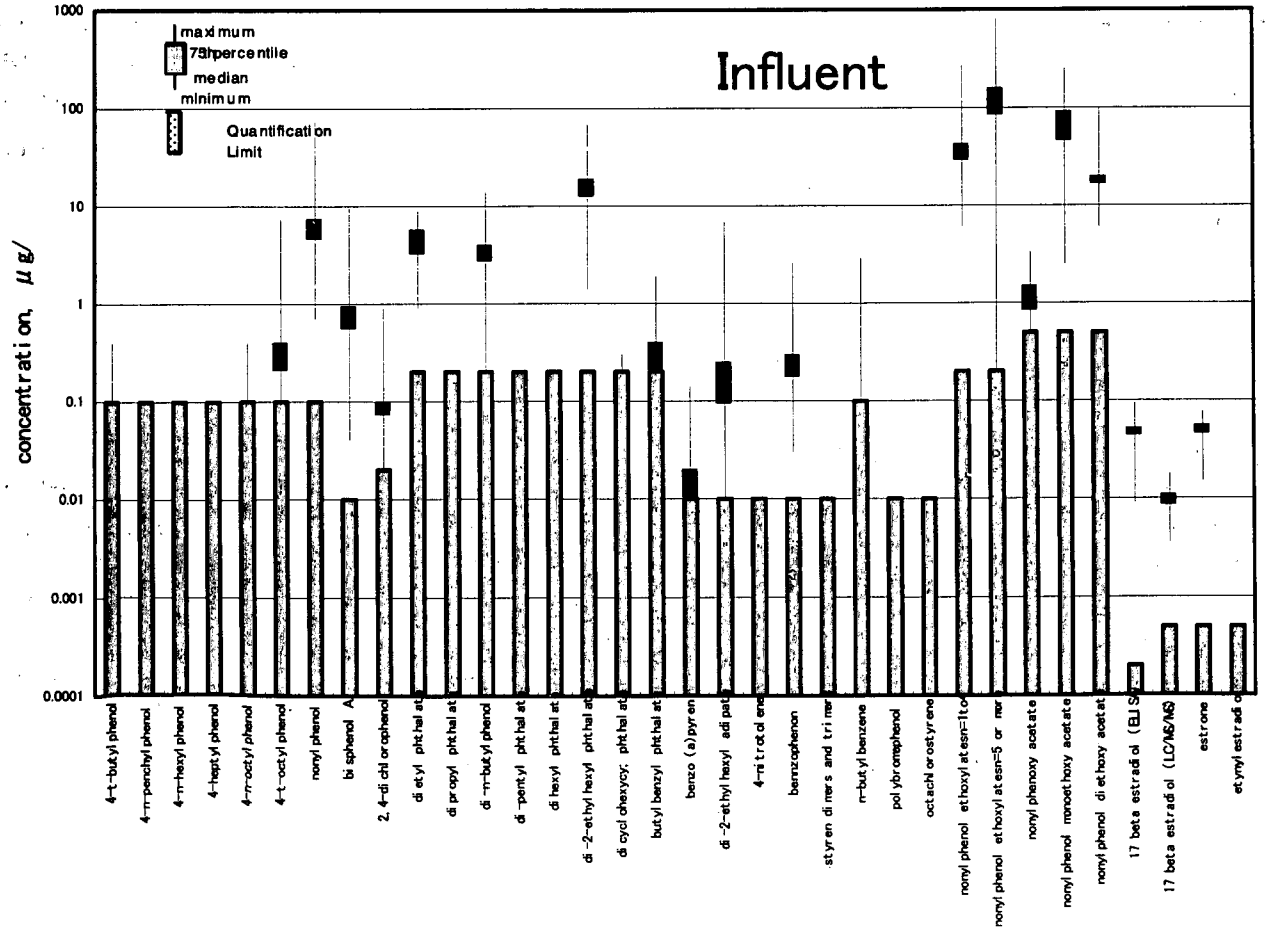


Fig. 2. Influent concentrations of suspected EDs expressed in terms of range and percentiles

3.1.2 Final discharge concentration

In the final effluent of the STPs studied, 18 COC were below their DLs at all the STPs, and 8 COC were above their QLs at least one of the STPs. The COC detected above their QLs include 4-*t*-octyl phenol, nonylphenol, bisphenol A, 2,4-dichlorophenol, di-*n*-butyl phthalate, di-2-ethylexyl phthalate, di-2-ethylhexyl adipate and benzophenone. Nonylphenol ethoxylates, nonylphenol ethoxy acetates, 17 β -estradiol (measured with the ELISA or LC/MS/MS methods) and estrone were found in either secondary effluent or final effluent of at least one of the STPs. Ethynyl estradiol was not detected in treated sewage at any STPs. Although the concentrations of nonylphenol in most of the final discharges were approximately 1 ì g/L, the related substances such as nonylphenol ethoxylates and ethoxy acetates existed at one ì g/L to few tens ì g/L. Based on the nonylphenol-equivalent unit (median value), nonylphenol occupies an insignificant fraction of total unit, while nonylphenol ethoxylates occupy 17% and nonylphenol ethoxy acetates constitute 83%. Among estrogens (i.e., estrogen and its related compounds) that were measured by LC/MS/MS, estrone occupies the most part of estrogens and 17 β -estradiol is minimal.

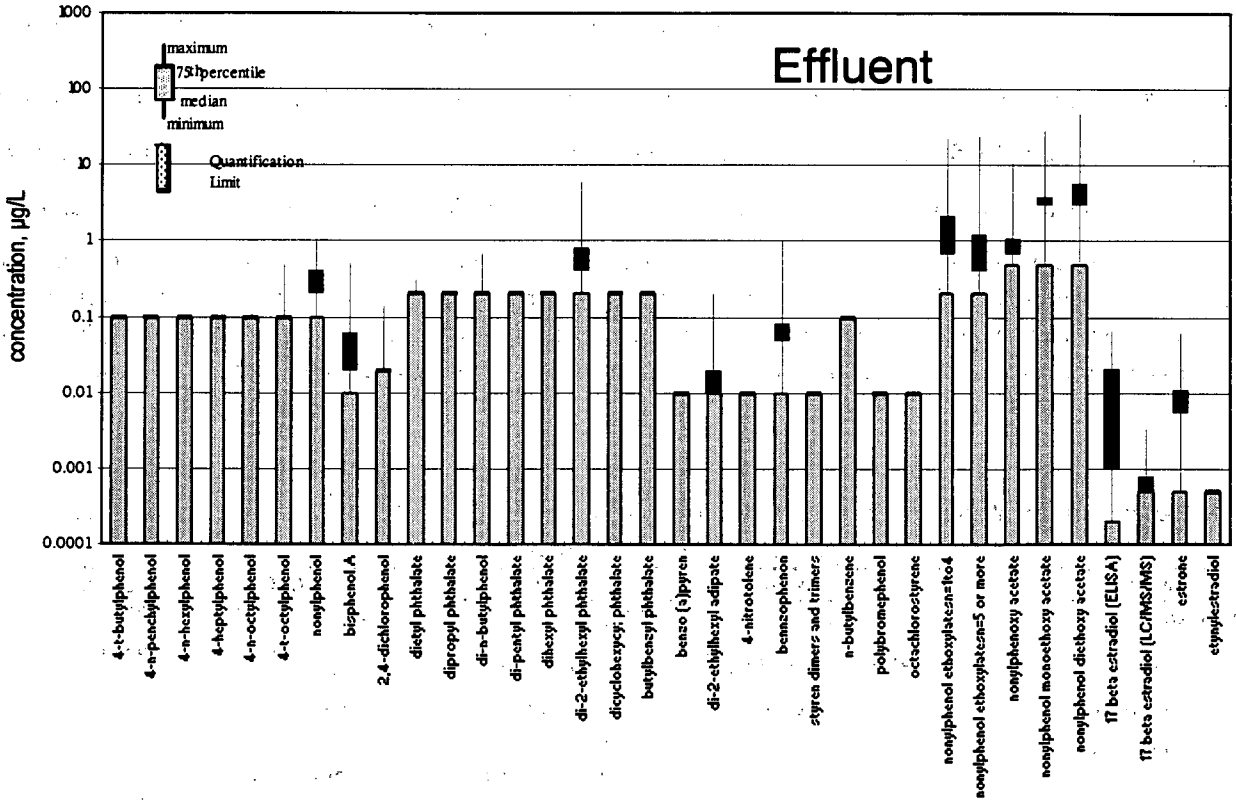


Fig. 3. Final effluent concentrations of suspected EDs expressed in terms of range and percentiles

3.2 Reduction of suspected EDs in sewage treatment plants

The relationship between the influent and effluent concentrations are shown in Fig. 4, in which only the substances with their median concentrations in the influent exceed their QLs are presented. The influent and effluent concentrations are plotted on the abscissa and ordinate, respectively. The linear line represents the removal efficiency (%) of COC. The plot that is closer to the abscissa indicates a greater reduction of COC in the STPs, and the plot closer to the 45-degree line indicates little or no reduction of the substance in the STP. The samples were collected via single grab sampling in the first year and composite sampling in the following two years. The plots show that the reduction trends for the both sampling cases are similar, and that, in some of the STPs, the effluent concentrations of 17 β -estradiol, estrone, and/or benzophenone are nearly the same or above their influent concentrations.

Although the concentrations of COC in influent (sewage) varied, they were consistently reduced in most of the STPs. To develop an understanding of the reduction trends for COC in the STPs, the available data are statistically analyzed and presented in Table 2. For the STPs in which the influent concentration exceeded its QL, we calculated range and median of the removal efficiency for each substance. A parenthesis in Table 2 is the removal efficiency calculated based on median of the influent concentrations and median of the effluent concentrations. The result indicates that most of COC in sewage can be removed in a STP with efficiencies greater than 90% (based on median values), and that STPs in Japan are effective in reducing most of the suspected EDs identified in this study. Three notable exceptions are benzophenone, 17 β -estradiol, and estrone. The removal efficiencies of these compounds (approx. 70-80%) are somewhat smaller than those of other compounds (>90%). Benzophenone, 17 β -estradiol (measured by ELISA or LC/MS/MS) and estrone are scattered in a wide range. At some of the STPs, negative removal efficiencies were obtained for 17 β -estradiol and estrone. This observation may suggest that 17 β -estradiol and estrone could increase in the treatment processes. Nonyphenol ethoxy acetates also tend to increase in the treatment. One plausible explanation for this trend is that these substances can be formed from conjugate-form of estrogens and nonylphenol ethoxylates. Further investigation at existing STPs and controlled experiments in pilot plants and laboratories can permit an explanation of the complex reactions occurring under various operating conditions.

Table 2. Range of removal efficiencies of the substances whose median concentrations exceeded the quantification limits in sewage treatment plants

Substances	FY 1998	FY 1999	FY 2000	Over all
Nonylphenol	76%～99%(94%)	84%～99%(98%)	>82%～>99%(-)	(97%)
Bisphenol A	30%～>99%(95%)	43%～99%(>96%)	>47%～>99%(>96%)	(96%)
2,4-dichlorophenol	50%～>98%(-)	60%～>95%(-)	no data	-
Di-ethyl phthalate	>83%～>98%(-)	>78%～>97%(-)	no data	-
Di-n-butyl phthalate	>75%～>98%(-)	>78%～>99%	no data no data	-
Di-2-ethylhexyl phthalate	61%～>99%(95%)	78%～>99%(>98%)	>47%～>98%(>94%)	97%
Di-2-ethylhexyl adipate	～>99%(-)	40%～99%(-)		-
Benzophenone	83%～>99%(65%)	～>98%(-)	>50%～>97%(89%)	71%
Nonylphenol ethoxylate (n=1～4)	66%～>99%(94%)	60%～>99%(98%)	52%～>99.5%(95%)	97%
Nonylphenol ethoxylate (n≧5)	83%～>99%(99%)	86%～>99%(99.5%)	>98%～>99.9%(-)	>99%
17β-estradiol(ELISA)	～>99%(67%)	～>99%	15%～>99%(79%)	77%
17β-estradiol(LC/MS/MS)	no data	no data	-160%～>96%(-)	-
estrone	no data	no data	-80%～>99%(86%)	86%

*When values in final discharge are below the detection limits (DLs), they are assumed to be the DLs and indicates ">XX%" . When values in final discharge are over DLs and below the quantification limits (QLs), they are assumed to be estimated concentrations. If the influent concentration is less than QLs, the data are not used for calculation of removal efficiency. In both the cases reduction efficiencies are expressed in more than (>) the calculated ratios. When the influent concentrations are below quantification limits, the reduction efficiencies are not calculated. "-" means the influent concentration is below QL or final discharge is below DL. "( )" means removal efficiency that is based on medians of influent and final discharge.

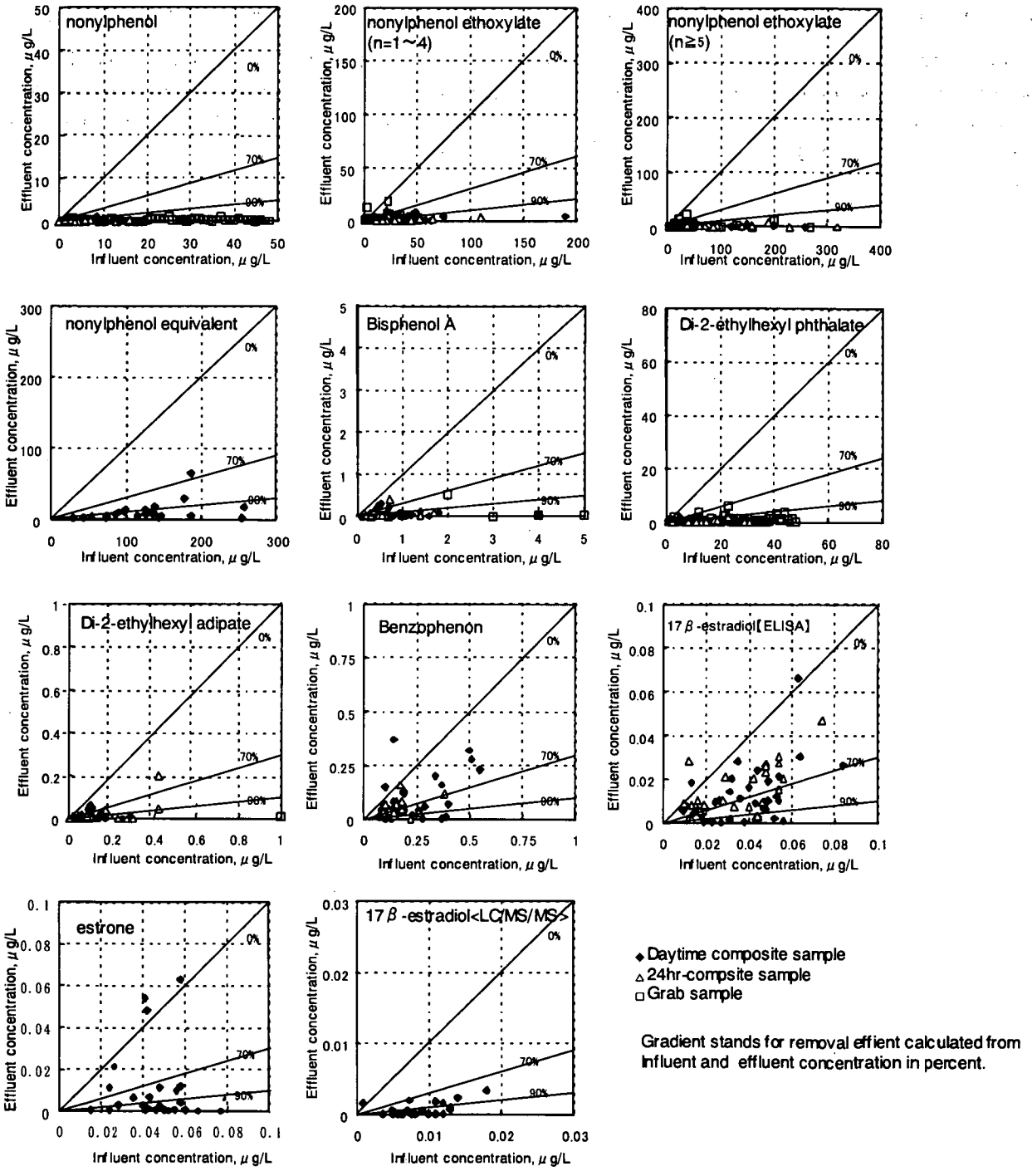


Fig. 4. Relationship between the influent and the final discharge concentrations of selected EDs in sewage treatment plants in Japan

3.3 Change in concentration during secondary treatment.

A typical STP consists of primary settling tanks, biological reactors (activated sludge process), secondary settling tanks, and disinfection units, in sequence. The suspected EDs in sewage, influent and effluent of the primary treatment, and final discharge were analyzed at the 34 STPs for 3 years. All the data obtained are statistically analyzed, and the 25^{th} and 75^{th} percentiles (rectangular box), ranges (error bar), and medians (black dot) are presented in Fig. 5. In the primary treatment, most of COC were reduced effectively, while some of them were reduced less effectively. For instance, benzophenone and 17 β -estradiol (measured using ELISA) exhibited little reduction in the primary treatment. On the other hand, all of COC were removed more effectively in the biological treatment (aeration tank) than in the primary treatment. Overall, the substances we investigated appear to be removed in the primary treatment and/or the secondary (biological) treatment. To gain a further understanding of their reduction mechanisms and fates, more detailed and extensive investigations in the filed and laboratory are necessary.

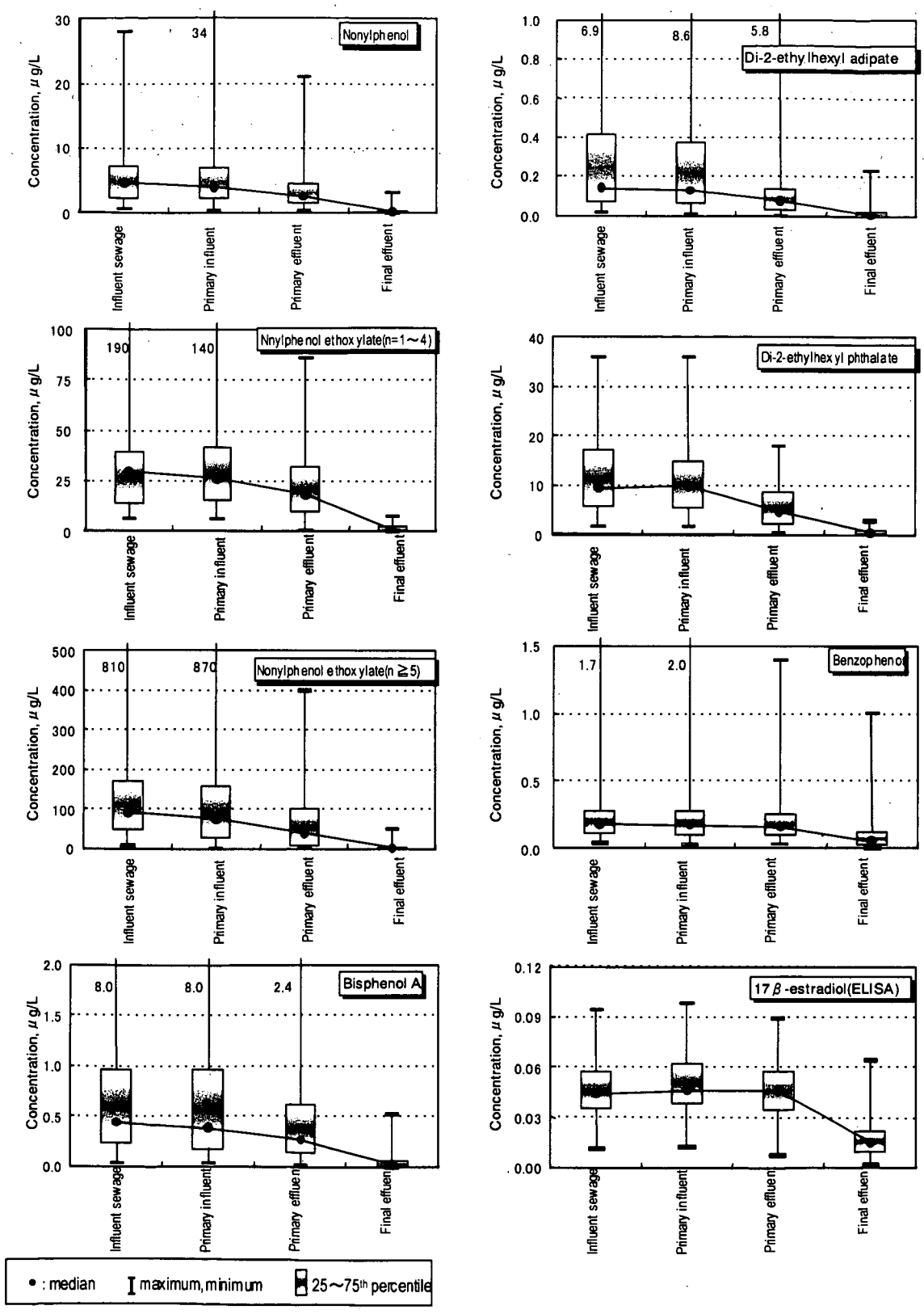


Fig. 5. Concentrations of suspected EDs in the sewage treatment plants.

Figs. 6 and 7 show plots of the removal efficiencies (%) against the aerated solids retention time (A-SRT) and the hydraulic retention time (HRT), respectively, for the suspected EDs. The plots reveal that the removal efficiencies of the concerned substances vary considerably in the STPs where A-SRT and/or HRT is small (approx. <15 days), and that the extent of the variation reduces to the higher efficiencies in the STPs with larger A-SRT (A-SRT >15 days) and/or HRT. Although a limited amount of data is available in the high A-SRT and HRT cases, this observation suggests that the biological treatment process (e.g., biological nitrogen removal process, oxidation ditch) with a large A-SRT or HRT is effective in removing EDs. Due to the limited available data and the differences in the STP's operating variables, this observation cannot be fully verified. Therefore, further studies are necessary.

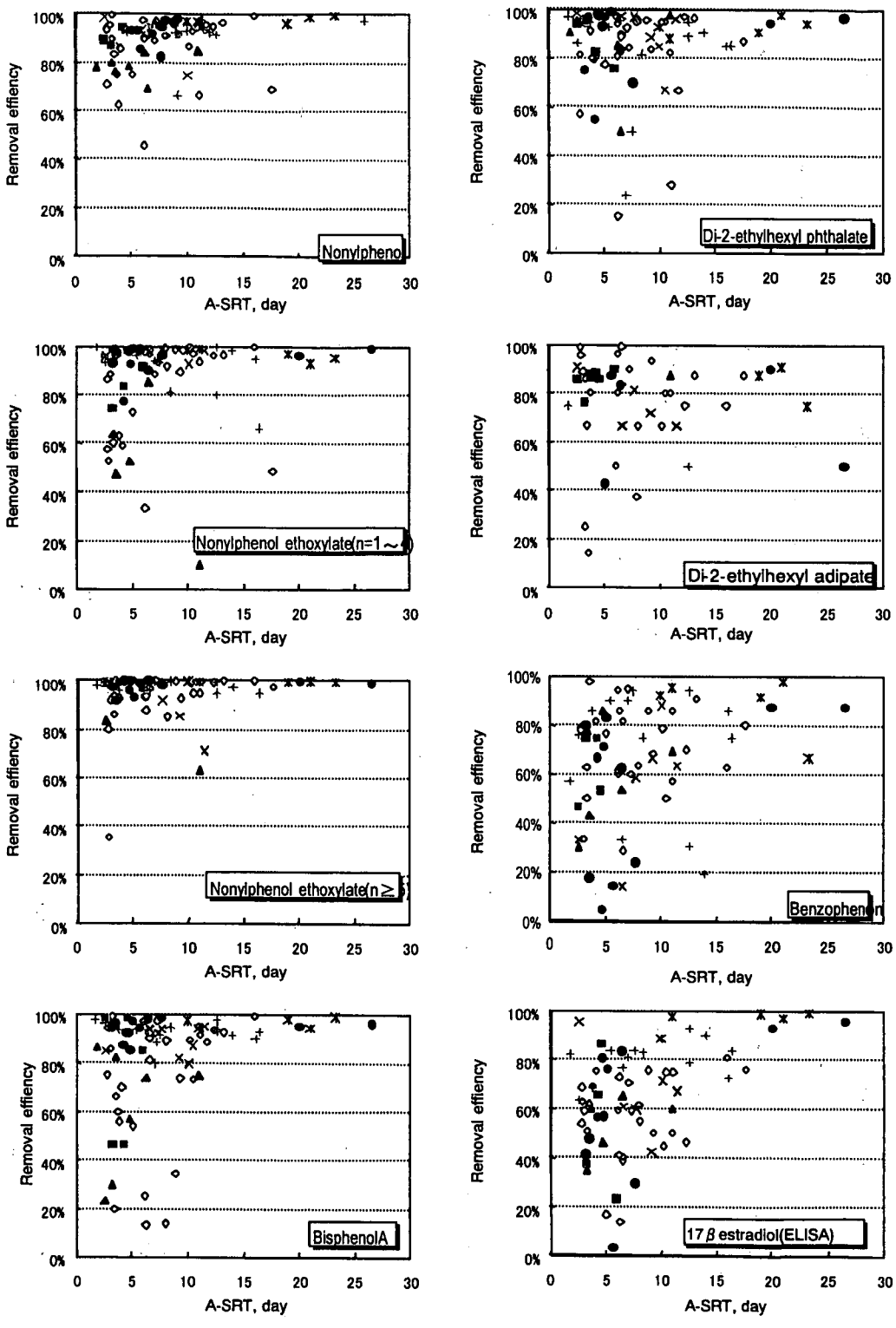
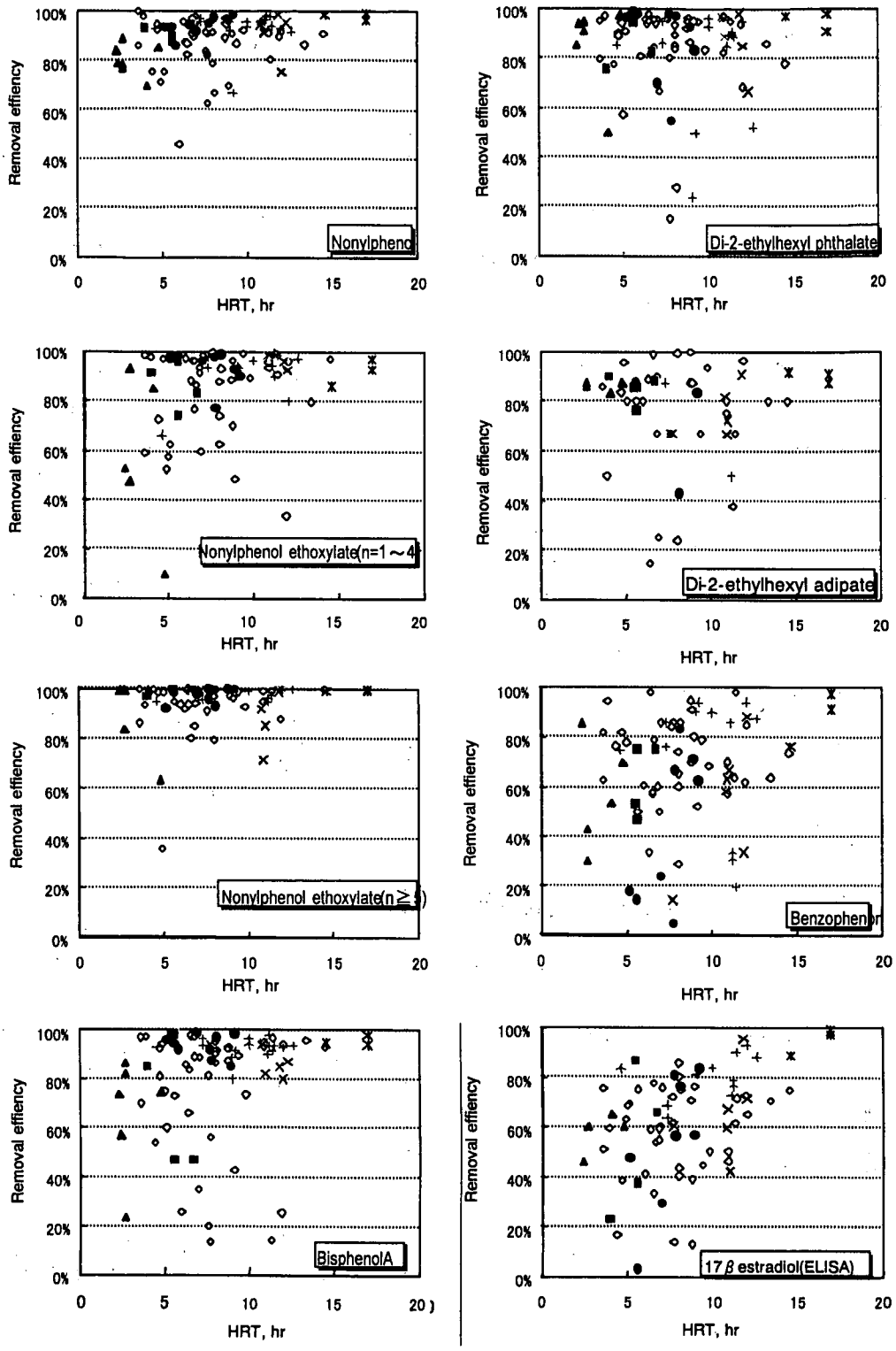


Fig. 6. Removal efficiencies of suspected EDs in the biological treatments with aerated sludge retention time (A-SRT)



◇:Conventional Activated Sludge(AS), ■:Step Aeration AS, ▲:Pure Oxygen AS, ×:Biological nitrogen removal AS,
＊:Oxydation ditch, ●:Biological phosphorus removal AS, ＋:Biological nitrogen phosphorus removal AS

Fig. 7. Removal efficiencies of suspected EDs in the biological treatments with hydraulic retention time (HRT)

3.4 Reduction in tertiary treatment

Tertiary treatment plants generally apply physicochemical processes following secondary (biological) treatment, aiming at wastewater reclamation and reuse. Thus, effectiveness of the tertiary treatment processes in removing EDs is of great concern. In this study, the physicochemical treatment processes following the biological treatment are evaluated by comparing the COC concentrations in the influent and effluent of each unit process. The processes evaluated in this study include rapid filtration, ozonation, activated carbon absorption, mechanical membrane filtration, and reverse osmosis (RO) membrane processes. As noted earlier, most of COC were below their QLs in the effluent of the biological process. Thus, only the substances that exceeded their QLs in the effluent of the biological process were chosen. Table 3 summarizes the available data using the number of samples that exceeded their QLs and the ranges of the concentrations in influent and effluent of each unit process applied in the tertiary treatment. Examination of Table 3 reveals that the sand filtration process contributes little in reduction of COC. The low COC removal may be resulted from the low suspended solids (SS) reduction by the sand filtration. Because SS in the secondary effluent is generally low, the removal of SS by the sand filtration is minimal. On the other hand, ozonation, activated carbon absorption, and RO membrane processes can contribute to some degrees of COC reduction as their concentrations in the effluent are somewhat lower than those in the influent of the process. Although many of COC are seen to be removed in the physiochemical treatment processes (following the biological treatment), the several compounds including benzophenone, nonylphenol ethoxylate, nonylphenol ethoxy acetates, 17 β -estradiol, and estrone tend to remain in the tertiary effluent above their QLs.

Table 3 Change of substance concentrations in specific physicochemical treatment processes after biological treatment

Unit: SS and COD are mg/L, the others are μg/L

substances	Quantification limit	Sand filtration		ozonation		Activated carbon absorption		Membrane Filtration		Reverse Osmpsis Membrane	
		influent	effluent	influent	effluent	influent	effluent	influent	effluent	influent	effluent
SS	1	<1～8 (27/30)	<1～4 (7/30)	<1～3 (2/7)	<1～1 (1/7)	<1 (0/2)	<1 (0/2)	<1 (0/1)	<1 (0/1)	- -	- -
COD	1	5.4～10 (13/13)	4.7～9.7 (13/13)	4.7～11 (15/15)	3.1～9.7 (15/15)	6.0～6.3 (2/2)	3.8～4.9 (2/2)	7.7 (1/1)	6.1 (1/1)	- -	- -
nonylphenol	0.3	n.d.～0.3 (1/30)	n.d.～tr(0.2) (0/30)	n.d.～0.6 (2/15)	n.d.～tr(0.2) (0/15)	n.d.～tr(0.2) (0/6)	n.d.～tr(0.1) (0/6)	n.d. (0/1)	n.d. (0/1)	n.d.～tr(0.1) (0/4)	n.d.～tr(0.1) (0/4)
bisphenolA	0.03	n.d.～0.33 (13/30)	n.d.～0.21 (8/30)	n.d.～0.21 (6/15)	n.d.～0.13 (1/15)	n.d.～0.11 (3/6)	n.d.～0.05 (2/6)	n.d. (0/1)	n.d. (0/1)	tr(0.01)～0.09 (1/4)	n.d.～0.04 (1/4)
di-2-ethylhexyl phthalate	0.6	n.d.～1.9 (10/30)	n.d.～2.8 (5/30)	n.d.～2.8 (4/15)	n.d.～0.7 (2/15)	n.d.～tr(0.5) (0/6)	n.d. (0/6)	tr(0.2) (0/1)	n.d. (0/1)	n.d.～1 (1/4)	n.d.～tr(0.4) (0/4)
di-2-ethylhexyl adipate	0.03	n.d.～0.05 (2/16)	n.d.～0.19 (2/16)	n.d.～0.03 (1/8)	n.d.～tr(0.02) (0/8)	n.d. (0/4)	n.d.～tr(0.01) (0/4)	- -	- -	n.d.～0.01 (0/3)	n.d.～n.d. (0/3)
benzophenon	0.03	n.d.～0.22 (8/15)	n.d.～0.13 (7/16)	tr(0.01)～0.13 (5/8)	n.d.～0.12 (2/8)	n.d.～0.12 (2/3)	n.d.～tr(0.02) (0/3)	tr(0.01) (0/1)	tr(0.02) (0/1)	n.d.～0.03 (1/2)	tr(0.01)～tr(0.02) (0/2)
nonylphenol ethoxylates (n=1～4)	0.6	n.d.～9.1 (9/14)	n.d.～10 (7/14)	n.d.～27 (2/6)	n.d.～1.0 (3/6)	n.d.～1.1 (1/3)	n.d.～tr(0.4) (0/3)	2.1 (1/1)	1.9 (1/1)	0.4～1.4 (1/2)	tr(0.4)～tr(0.5) (0/2)
nonylphenol ethoxylates (n≧5)	0.6	n.d.～3.0 (2/14)	n.d.～1.6 (2/14)	n.d.～1.0 (2/6)	n.d.～tr(0.3) (0/6)	n.d～tr(0.3) (0/3)	n.d.～n.d. (0/3)	n.d. (0/1)	n.d. (0/1)	n.d.～tr(0.2) (0/2)	n.d.～tr(0.3) (0/2)
nonylphenoxy acetate (NP1EC)	1.5	n.d.～3.8 (2/8)	n.d.～4.3 (1/8)	n.d.～4.8 (1/4)	n.d.～tr(0.8) (0/4)	n.d.～4.3 (1/2)	n.d.～3.6 (1/2)	tr(1) (0/1)	tr(1) (0/1)	- -	- -
nonylphenol monoethoxy acetate (NP2EC)	1.5	tr(1.4)～5.6 (7/8)	tr(0.8)～7.4 (6/8)	tr(1.3)～22 (3/4)	n.d.～2.3 (2/4)	n.d.～5.9 (1/2)	n.d.～5.1 (1/2)	3.8 (1/1)	3.2 (1/1)	- -	- -
Nonylphenol diethoxy acetate (NP3EC)	1.5	tr(0.8)～7.8 (7/8)	1.9～11 (8/8)	1.9～19 (4/4)	tr(1.0)～4.3 (2/4)	1.9～4.0 (2/2)	tr(1.1)～3.4 (1/2)	6.7 (1/1)	5.6 (1/1)	- -	- -
17β-estradiol (ELISA)	0.0006	0.0012～0.064 (24/24)	n.d.～0.044 (22/24)	n.d.～0.029 (12/13)	n.d.～0.019 (4/13)	n.d.～0.023 (4/6)	n.d.～0.0082 (2/6)	0.026 (1/1)	0.021 (1/1)	0.0012～0.021 (4/4)	n.d.～n.d. (0/4)
17β-estradiol (LC/MS/MS)	0.0015	n.d.～tr(0.0014) (0/8)	n.d.～0.0025 (1/8)	n.d. (0/4)	n.d. (0/4)	n.d.～0.0025 (1/2)	n.d. ～tr(0.0008) (0/2)	n.d. (0/1)	n.d. (0/1)	- -	- -
estron (LC/MS/MS)	0.0015	tr(0.0009) ～0.081 (5/8)	n.d.～0.05 (3/8)	tr(0.0011) ～0.0042 (3/4)	tr(0.0005) ～tr(0.0014) (0/4)	tr(0.0008) ～0.05 (1/2)	0.0019～0.01 (1/2)	tr(0.0013) (0/1)	tr(0.0011) (0/1)	- -	- -

( ) stands for sample number exceeding quantification limit／total sample number

"-" stands for no available data

3.5 Sludge treatment processes

The concentrations of COC in sludgs from various processes are summarized in Table 4. The concentrations are expressed in terms of mass of COC per unit mass of dry solid (ì g/g). Note that the data sizes differ greatly with varying processes and COC. The COC concentrations in excess sludge from the biological treatment tend to be lower than those in primary sludge. The median concentrations of COC in the incinerated ash are below their DLs, and are the lowest in any sludge types compared herein. It is noteworthy that the median concentration of nonylphenol is larger after the digestion process than the thickening process. As the data are compared among the STPs that apply an anaerobic digestion process alone, the increase in nonylphenol in the digested sludge is apparent. The result supports the previous observations[15] that nonylphenol was produced under the anaerobic conditions.

Table 4 Change of substance concentrations in sludge during sludge treatment processes

	nonylphenol	bisphenol A	di-2-ethylhexyl phthalate	di-2-ethylhexyl adipate	benzophenone	nonylphenol ethoxylates (n=1to 4)	nonylphenol ethoxylates (n≧5)	17bete estradiol
Primary Sludge								
range	0.7 - 130	nd – 1.5	3.4 - 440	nd - 1.8	nd – tr(0.30)	nd - 210	nd - 74	nd - tr(0.049)
median	6.1	tr(0.28)	86	nd	nd	28	17	tr(0.019)
quantification rate	25/25	6/25	25/25	1/25	0/25	24/25	23/25	0/25
Excess Sludge								
range	nd - 41	nd - 0.63	nd - 230	nd - 7.3	nd	nd - 120	tr(0.3) - 17	nd - 0.12
median	1.4	nd	55	nd	nd	10	2.3	tr(0.023)
quantification rate	36/62	2/62	26/29	1/29	0/29	28/29	23/29	7/29
Condensed Sludge								
range	nd - 64	nd - 0.98	3.8 - 150	nd - 3.1	nd - tr(0.29)	nd - 120	nd - 29	nd - 0.15
median	3.3	tr(0.24)	110	nd	nd	12.0	2.8	tr(0.032)
quantification rate	16/18	3/18	17/17	2/17	0/6	16/17	16/17	6/17
Digestion Sludge								
range	13 - 210	tr(0.22) - 3.2	nd - 200	nd - 1.0	-	tr(0.9) - 21	tr(0.4) - 13	tr(0.020) - 0.064
median	38	0.92	65	nd	-	14	1.8	tr(0.038)
quantification rate	7/7	5/7	6/7	1/7	-	6/7	5/7	2/7
Dewatered Sludge								
range	tr(0.17) - 210	nd - 1.2	nd - 170	nd - tr(0.70)	nd - tr(0.42)	nd - 47	tr(0.3) – 71.0	nd - 0.062
median	6.0	tr(0.29)	97.0	nd	nd	14	9.0	tr(0.008)
quantification rate	21/23	7/23	17/18	0/8	0/8	17/18	16/18	4/18
Incinerated Ash								
range	nd - 0.57	nd	nd	nd	nd	nd	nd	nd
median	nd	nd	nd	nd	nd	nd	nd	nd
quantification rate	1/19	0/19	0/16	0/16	0/6	0/14	0/16	0/16

4. Results of estrogen-like activity

4.1 Estrogenicities in influent and effluent of STPs.

The estrogen-like activities (estrogenicities) of the influent sewage and the final effluent are compared in Fig. 8. The estrogen-like activities of the influent and final discharge of the 34 STPs were measured and expressed in terms of 17 β -estradiol equivalent.[12,13] Total number of samples measured is 98. The estrogen-like activity of the influent ranges from 0.0099 to 0.82 ì g/L, and its median and mean are 0.077 ì g/L and 0.099 ì g/L, respectively. On the other hand, the estrogen-like activity of the final discharge ranges from 0.0001 to 0.21 ì g/L, and its median and mean are 0.0073 and 0.018 ì g/L, respectively. The removal efficiency ranges from -119% to >99%, and its median and mean are 90% and 91%, respectively. Four samples collected in 3 STPs gave negative removal efficiencies because the estrogen-like activities of the final discharge are higher than those of the influent. Because these samples (from the 3 STPs) were collected as grab samples in the first year, the negative values might not give representative efficiencies occurring in the STPs. The estrogen-like activity was, in general, reduced effectively in most of the STPs studied.

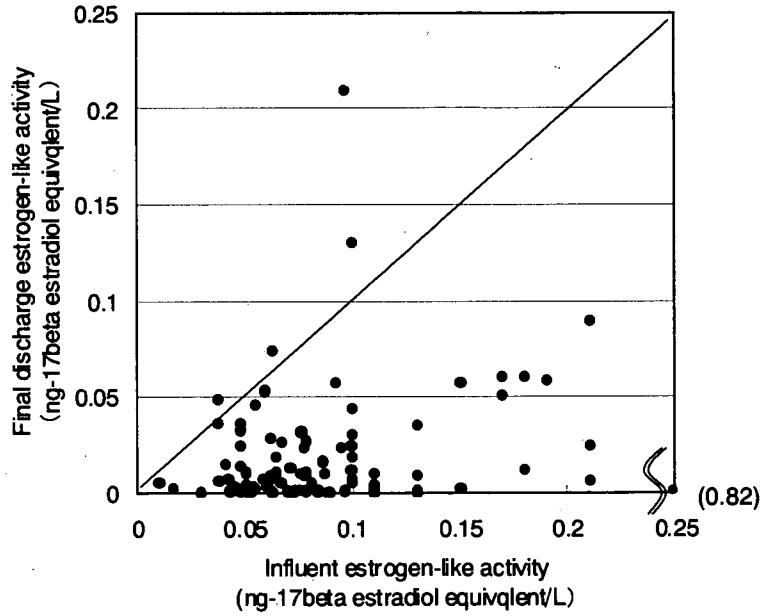


Fig. 8. Estrogen-like activities as 17 β -estradiol equivalent (ng/L) in influent sewage and final discharge

4.2 Change in estrogenicities in sewage treatment.

Estrogenicities of untreated, partially treated, and treated wastewater were investigated at nine STPs. Samples were taken from the sewage influent, influent and effluent of the primary treatment, effluent of secondary treatment (activated sludge process), and final effluent after disinfection. The samples were analyzed for estrogenicity, and results are summarized in Fig. 9 in terms of the range, the 25th and 75th percentiles, median, and average. Three outlying values were removed from the statistical analysis, but are presented in Fig. 9. As is seen, the primary influent samples exhibited the greater estrogenicity than the influent sewage samples. The greater estrogenicity is probably due to the supernatant returned from the sludge treatment process. Note that some of the STPs studied have relatively large sludge treatment facilities that even treat sludge from other STPs in the same municipalities. The estrogenicity in the effluent of the primary treatment is somewhat lower than that in its influent, indicating that the primary treatment can

reduce the estrogenicity to some extent. A dramatic decrease in the estrogenicity is observed in the secondary treatment process in which all the STPs studied use the activated sludge process, except for the 3 STPs which gave 3 outlying values. The results suggest strongly that the estrogenicity can be effectively reduced in the biological treatment processes. A slight increase in the estrogenicity is seen in the final effluent. Because this analysis is based on the data obtained from a single grab sampling occasion (at each sampling location), there is considerable uncertainty concerning interpretation of the results.

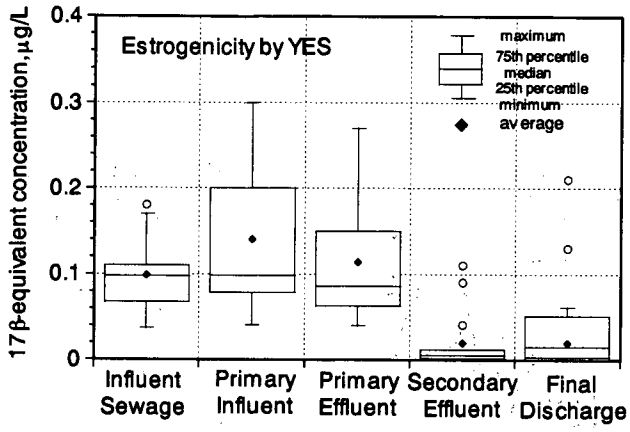


Fig. 9. Change of the estrogenicity in nine sewage treatment plants

5. Discussion

5.1 Gap between estrogen-like activity measured with YES and chemical analysis

In the previous studies,[12,13] the estrogenic potential by individual substance was measured. In this study, we used the estrogen potential derived in their studies to estimate the estrogen-like activities for the substances concerned in the nationwide study.[16] It is assumed that each substance exhibits estrogenic effect on the yeast independently from other substances, and that the effects by multiple substances are additive. These assumptions are supported by Miyamoto *et al.* (unpublished data). We calculated total estrogen-like activity for each substance based on its estrogenic potential (expressed in terms of 17 β -estradiol equivalent) and the concentration of the substance monitored in the nation-wide survey, and called it "theoretical estrogenicity".[12,13] If the estrogen-like activity obtained by the YES assay adequately expresses total amount of estrogen-like substances, the YES value is generally greater than the estrogenicity calculated using all the substances found in the nation-wide survey. If the relative estrogenic potential (REP) of 17 β -estradiol is assumed to be 1, REP for the substances monitored in the nation-wide survey[16] are given as follows: Estrone 0.3; 17 β -ethynylestradiol 0.5; 4-tert-butylphenol 0.00002; 4-n-pentylphenol 0.0007; 4-n-hexylphenol 0.0006; 4-n-heptylphenol 0.00006; 4-n-octylphenol 0.000005; nonylphenol 0.001; 4-t-octylphenol 0.02; bisphenol A 0.00006; 2,4-dichlorophenol 0.000002; benzophenone 0.0000006; others have no significant positive data at the concentrations up to 2 g/L.[12,13]

Fig. 10 shows the theoretical estrogenicities of 17 β -estradiol and other suspected EDs along with the observed estrogenicies (measured using the YES assay) for the substances monitored in the nation-wide survey.[16] The samples are numbered in increasing order of the estrogen-like activity. As seen in the samples with the low

estrogen-like activities (<I15, <F23), the calculated estrogenicity of 17 β -estradiol accounts for a large portion of the total estrogenicity, leaving little or no room for other ED substances observed in the nation-wide survey. This trend is seen in the both influent sewage and final discharge cases, and supports that the ELISA method may overestimate the concentration of 17 β -estradiol in wastewater as was reported in our previous study.[12,13] On the other hand, in the samples with the larger estrogen-like activity (>I15, >F23), the observed estrogenicity (the YES assay) is considerably larger than the corresponding theoretical value particularly in the influent sewage. The gap suggests the presence of unknown chemicals that are not accounted as substances that would cause estrogenicity.

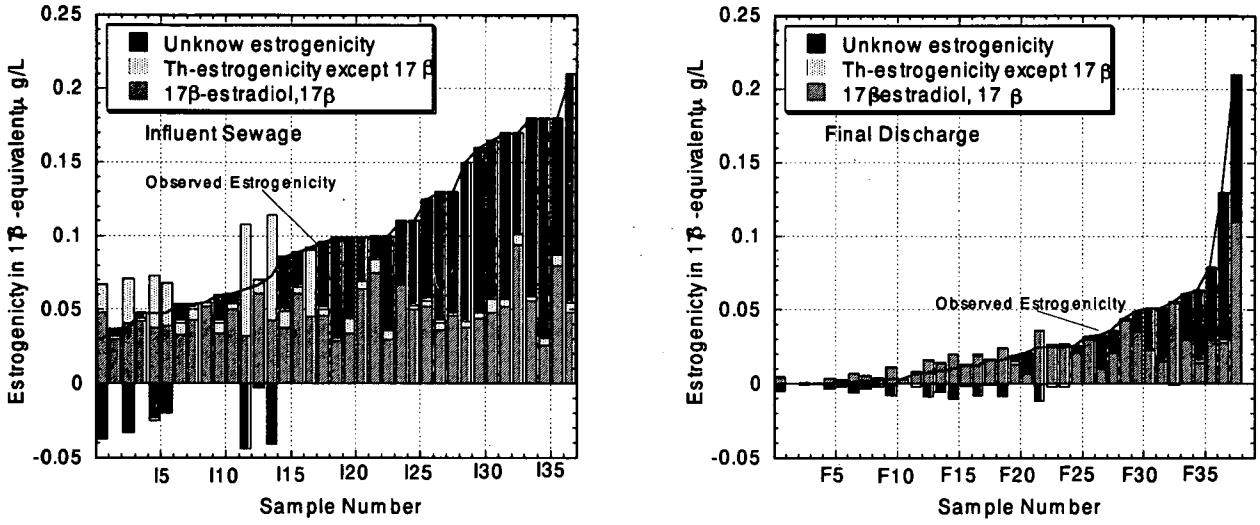


Fig. 10. Estrogen-like activities of influent sewage and final discharge of STPs, calculated based on estrogen data collected using ELISA[13]

In the third year, 13 samples of the influent sewage and the final discharge were analyzed for 17 β -estradiol and estrone using LC/MS/MS, in place of the ELISA method. The theoretical estrogenicity was calculated based on the analytical data, and compared with the observed estrogen-like activity (measured using the YES assay). Results of this comparison (Fig. 11) reveal that the observed estrogenicity is larger than the theoretical value in all the influent sewage and final discharge samples except one. Estrone occupies the largest portion of total estrogenicity as compared to other substances. Based on the average data of the 13 samples, the following observations can be made. In the influent sewage, 17 β -estradiol, estrone, and other measured chemicals (particularly nonylphenol) contribute, respectively, 10%, 15%, and 3% of total estrogenicity, and the estrogen-like activity derived from unidentified substances accounts for more than 70% of total activity. On the other hand, in the final discharge, 17 β -estradiol, estrone, and other measured chemicals (particularly nonylphenol) contribute 2.5%, 22% and 0.5%, respectively, but the estrogen-like activity derived from unknown substances occupy more than 75% of the estrogen-like activity in the influent sewage.

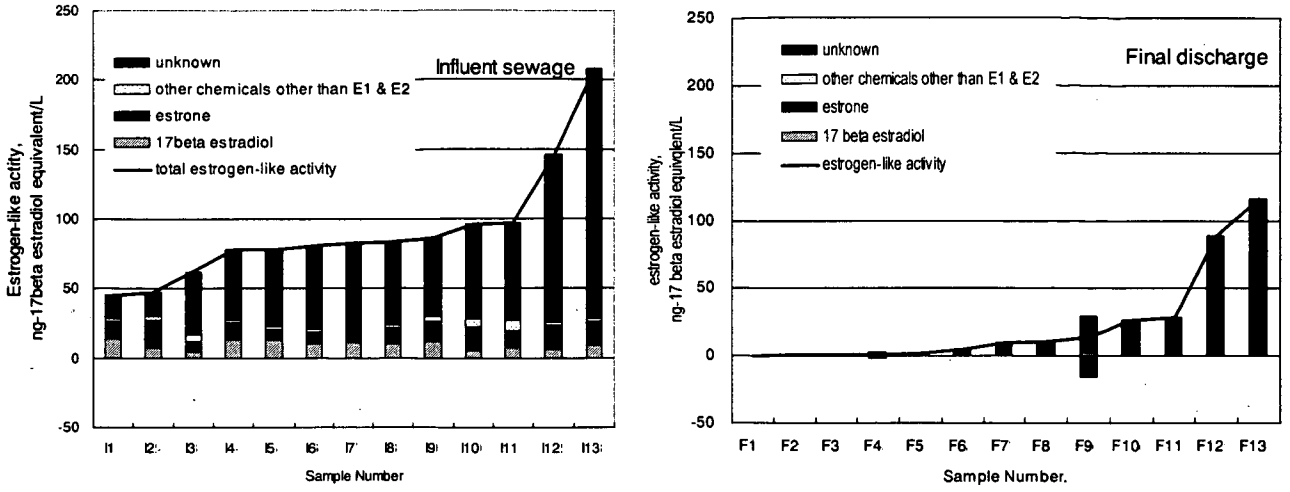


Fig. 11. Estrogen-like activities of influent sewage and final discharge of STPs, calculated based on estrogen data collected using LC/MS/MS (from third year study)

5.2 Gap of 17 β-estradiol (E2) concentration between ELISA method and LC/MS/MS

Recent studies revealed that the concentrations of 17 β-estradiol in sewage and treated sewage analyzed using LC/MS/MS is less than 10% of those analyzed with the ELISA method. [20][21] The final report released by MLIT in cooperation with Public Works Research Institute (PWRI) and the local governments also revealed the large discrepancy between the 17 β-estradiol concentrations analyzed with the ELISA method and with the LC/MS/MS methods.[16] The 17 β-estradiol ELISA kit used in the nationwide study is reported to rarely cross-react with estrone (Assay Designs, unknown) that is considered to make little contribution to the value measured with the ELISA kit. Statistically speaking, however, there is a weak relationship between 17 β-estradiol concentration measured with the ELISA kit and the estrone measured with LC-MS/MS [16]. In our study, the 17 β-estradiol measurement by the ELISA kit caused a positive error in the presence of linear alkylbenzene sulphonate (LAS) at 1000 mg/L; thus, the concentrated LAS in an extract from sewage is thought to be one of the factors that interfere with the ELISA measurement. To remove LAS from an extract, the SPE method was modified; that is, eluate methanol was replaced by dichloromethane. In the modified method, LAS was not eluted from C-18 SPE column while 17 β-estradiol was eluted thoroughly with dichloromethane. When comparing the 17 β-estradiol (E2) concentrations measured with LC/MS/MS and the ELISA method, the concentrations of E2 by ELISA with dichloromethane (as eluate) gave less deviation from those with LC/MS/MS than the concentrations of E2 by ELISA with methanol. The result suggests that the methanol eluate extracts in the previous studies might have contained interfering, hydrophilic substances such as LAS.

Many E2-ELISA kits are commercially available, but their differences are unknown when they are applied to reqal sewage samples. Therefore, the comparisons were made on following products: Assay Design (E2-AD), Takeda

(E2-TK), Cayman Chemicals (E2-CC), Neogen (E2-NG), and R-Biopharm (E2-RB). For the comparison, 28 samples from various locations of the STP were analyzed with each kit under the same conditions. The extraction column C-18 SPE and eluate dichloromethane were used. Results are presented in Table 5, from which it is seen that all the kits overestimated the concentrations of 17 β -estradiol. Nevertheless, the differences between the LC/MS/MS method and E2-TK ELISA was smallest.

Table 5 Average 17 β -estradiol concentrations in a sewage treatment plant by LC-MS/MS and commercially available ELISA kits [14)]

ng/L

Sample	n	LC-MS/MS	E2-AD	E2-TK	E2-CC	E2-NG	E2-RB
primary effluent	10	8.4	21.1	12.5	81.4	97.6	202.2
aeration tank	13	1.9	4.4	2.1	9.4	7.8	31.6
secondary effluent	5	1.2	5.3	2.6	15.1	18.8	75.9

5.3 Origin causing estrogenic effects in sewage and treated sewage

What causes estrogen-like activity in sewage or treated sewage? Our previous study suggested the existence of unidentified substances that have a potential to cause estrogen-like activities in sewage, treated sewage, and river water receiving treated sewage. There is strong evidence that estrogen-like activity measured with YES was greater than the estrogen-like activities estimated from concentrations of suspected EDs and their estrogenic potentials. In an effort to identify these substances, we examined the spectrum of estrogen-like activities in sewage and treated sewage using the polarity fractionation method. The fractionation method was carried out as follows:

1) One liter of a sample was pre-filtered with a glass fiber filter, GF/B. Then the filtrate was extracted with a C18 solid-phase extraction (SPE) cartridge and was eluted with 10 ml of methanol. The suspended matters on the GF/B were supersonic-extracted twice with 15 ml of methanol. The methanol eluate and the methanol extract were mixed and dried. The residual was solved in 1 ml of hexane (Hex)/dichloromethane (DCM) (50:50, v/v).
2) The Hex/DCM solvent was passed through a silica-gel cartridge (SepPack-silica) that had been conditioned with acetone (Ace) and a mixture of Hex: DCM (1:1) in order.
3) Nine different polar solvents were prepared by mixing hexane (Hex), dichloro-methane(DCM), acetone(Ace), and methanol (MeOH) as shown in Table 6. The silica-gel cartridge was eluted with each eluate one by one. Then, nine eluate fractions were collected and were named F1 to F9. Each fraction from F1 to F9 was dried and was dissolved again in 0.1 ml of dimethylsulfoxide (DMSO) solution.

Table 6 Eluates used for nine fractionations and distribution of selected COC concentrations

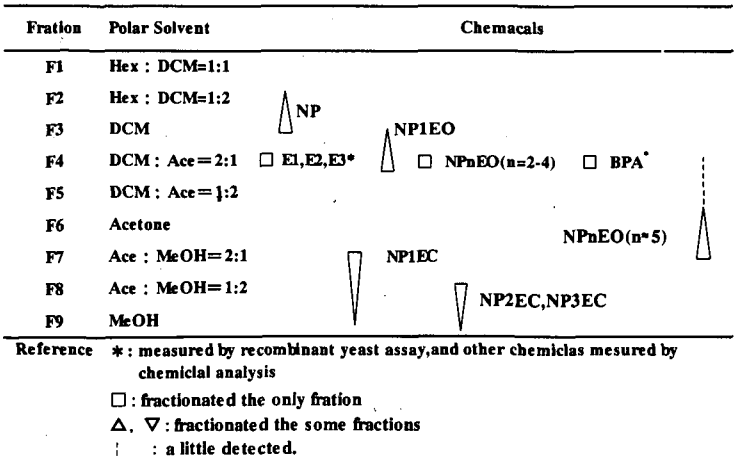


Table 6 indicates which fractionation includes selected COC, suspected EDs and related substances, in the water environment. After influent sewage and effluent sewage were fractionated with the above method, the estrogen-like activity of each fraction was measured using YES. The results based on influent sewage and secondary effluent of 22 STPs (shown in Fig. 12) indicate that the estrogen-like activity is widely distributed from F1 fraction (low polarity) to F9 fraction (high polarity) ranges, for the influent sewage. This finding suggests the existence of unknown substances other than estrone, 17β-estradiol, and nonylphenol in the influent sewage. However, the estrogen-like activity in both F2 and F4 fractions were identified in all the influent sewage samples. Particularly, F4 fraction in both the influent sewage and secondary effluent occupied the largest portion among nine fractions in general, and F2 fraction has the second largest fraction. The estrogen-like activity in other fractions varies with the STPs. In F2 fraction, nonylphenol contributed largest portion to the total estrogenicity for both the influent sewage and secondary effluent. In F4 fraction, estrone and 17β-estradiol are estimated to contribute on average 35% and 6%, respectively, to the total estrogenicity of the influent sewage, while they occupy, respectively, 72% and 4% of the total estrogenicity of the secondary effluent. The result implies that there exist more estrogen-like substances in addition to estrone and 17β-estradiol.

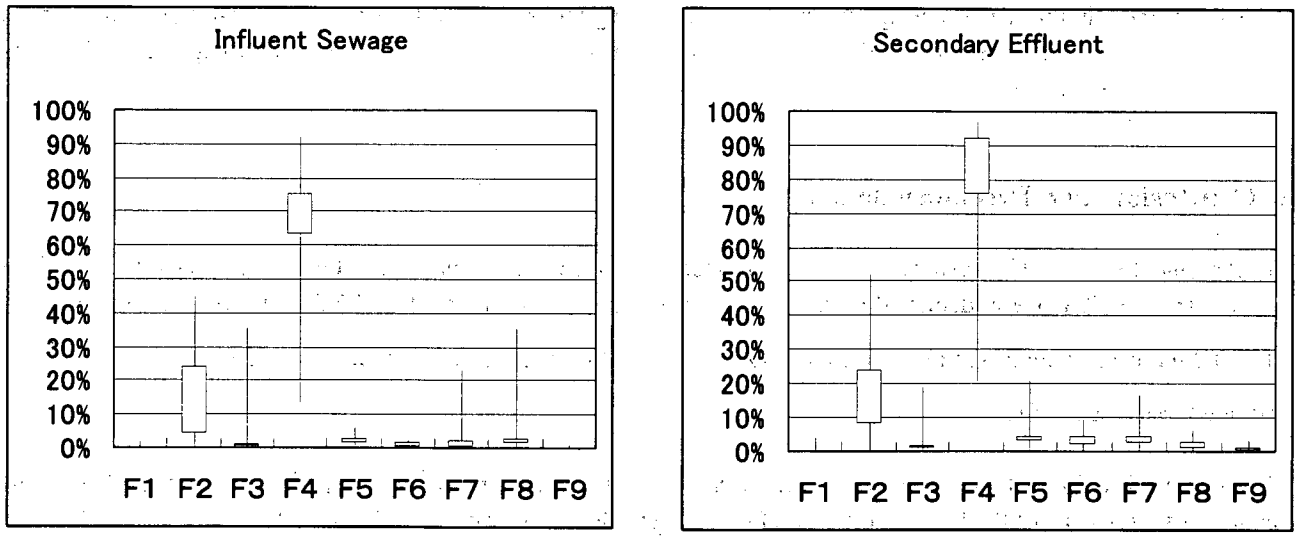


Figure 12 Spectrum of estrogenicities in nine fractions in influent sewage and secondary effluent in 22 sewage treatment plants (range and the 25th and 75th percentiles)

5.4 Carp breeding test – male carp exposed to secondary effluent of a sewage treatment plant

As discussed above, we found that estrogen-like activity exists in sewage discharge although the activity tends to decrease to a large extent in STPs. However, it is not clear if residual estrogen-like activity of STP effluent causes feminization of fish living in their receiving rivers in Japan. UK researchers reported that 1 to 10 ng/L of 17β-estradiol, 25 to 50 ng/L of estrone, and 20 ug/L of nonylphenol cause induction of vitellogenin in male rainbow trout [4]. Japanese researchers also reported that 8 ng/L of 17β - estradiol induces vitellogenin [17] and 5 ng/L of 17β-estradiol produced vitellogenin-like protein in male Medaka[18]. Based on such recent studies, remaining estrogens or estrogen-like substances in final discharge from STPs might induce vitellogenin in male fish in the receiving water. The field survey by the MLIT reported that a quarter of wild male carp caught in ten rivers have some vitellogenin in their blood, but the cause of vitellogenin in male carp is unclear[19].

In our carp breeding experiments, male carp were exposed to secondary effluent of a STP. The first experiment was conducted in the early spring of Year 2000. Four male carp out of five increased their serum vitellogenin concentrations that were not observed prior to their exposure to the effluent. The vitellogenin concentration in carp began rising soon after their exposure, and reached to stable levels in four weeks. Eight weeks later, the secondary effluent was changed to dechlorinated tap water. In three weeks, their serum vitellogenin concentrations decreased to the same levels as those in the start of the experiment. An average estrogen-like activity of the effluent measured with YES was 4.9 ng $\pm$ 0.8 ng E2 equivalent /L. In the control test using dechlorinated tap water, no serum vitellogenin was confirmed in any male carp. Therefore, the synthesis of vitellogenin in male carp might be reversible depending on the levels of estrogen-like substances, particularly with the estrogen-like activities of the STP effluent we tested.

Two more experimental runs were carried out at the same STP in the summer of Year 2000 and from February to April in 2001. However, any significant increase in the serum vitellogenin concentration could not be observed in the male carp that were exposed to the effluent. Averages of the estrogen-like activities in the effluents measured by YES were 3.7 ng $\pm$ 0.4 ng E2-equivalent/L in the summer experiment, and 7.0 ng $\pm$ 1.4 ng E2-equivalent/L in the winter-spring experiment. Considering the minimal differences of the estrogen-like activities in the effluent in all the experiments, the timing of exposure might be an important factor to explain the inconsistent phenomenon we observed.

6. Conclusions and Recommendations

(1) Among the 34 COC (suspected EDs and related substances) examined, 18 COC were detected in the influent sewage and 13 COC were identified in the final discharge of at least one of the 47 STPs studied. In these STPs, most of COC were effectively removed from sewage except for 17β-estradiol, estrone, and benzophenone, which were removed less effectively.

(2) Greater reductions of COC were observed in the secondary treatment than in the primary treatment. The degree of reduction varies with COC. In some of the STPs, the higher levels of certain COC were observed in the influent of the primary treatment than in the influent sewage

(3) We observed that, in many instances, levels of the most of COC were smaller in the excess sludge than in the primary sludge. The COC concentrations in the incinerated ash were below their QLs (due to combustion). The elevated levels of nonylphenol and bisphenol A were observed in the digested sludge.

(4) The advanced treatment processes, particularly combination of ozonation, activated carbon adsorption and/or RO membrane process, reduced the COC concentrations effectively. The processes including sand filtration, ozonation, RO membrane, and activated carbon absorption, removed 17β-estradiol (measured with the ELISA method) and benzophenone ,effectively. The both compounds were poorly removed in the biological treatment.

(5) Because the ELISA method tends to overestimate the concentration of 17β-estradiol in the influent sewage and in the final discharge, estrone may exist more than 17β-estradiol in final discharge. The usage of dichloromethane (DCM) as eluate in the SPE elution step can decrease the magnitude of overestimation of 17β - estradiol in sewage

samples. Based on the comparisons between the methods using the commercially available ELISA kits against the LC/MS/MS method, the E2 concentrations measure by E2 ELISA kit (Takeda) exhibited the smallest differences from those measured by LC/MS/MS.

(6) The estrogenicity of influent sewage was reduced to a large extent in the activated sludge process. In the study with the 13 STPs selected, we found that, in the influent sewage, 17β-estradiol and estrone account for most of the theoretical estrogenicity estimated from the estrogenic potentials of COC and their concentrations. The comparisons between the estrogen-like activity and the theoretical estrogenicity suggest that 17β-estradiol and estrone occupy approximately one quarter of the observed estrogenicity, and that significant amounts of unidentified estrogenic substances exist in influent sewage and final discharge, in addition to the substances monitored in the nation-wide study.

(7) The fractionation technique was applied in order to investigate the causes of estrogen-like activity in sewage and secondary effluent of the 22 STPs. We found that two major fractions (i.e., F2 and F4) contribute to the total estrogenicity of the influent sewage and of the secondary effluent. The fraction F4 coincided with estrone and 17β-estradiol, and occupied the largest portion of the activity, while the fraction F2 coincided with nonylphenol. These findings imply that nonylphenol, estrone, and 17β-estradiol are the major substances that cause the estrogen-like activity. Because some of other fractions also exhibited estrogen-like activity, other estrogen-like substances may exist in the fraction F4.

(8) We conducted experiments in which male carp were exposed to the secondary effluent of the STP. In the first run, the level of serum vitellogenin was increased in 80% of the male carp in four weeks, and the vitellogenin level decreased to the same levels as the beginning of the exposure. Although two additional experiments were conducted, no increase in serum vitellogenin in male carp was observed. Further investigation is necessary to determine if estrogen-like substances in treated sewage cause fish to be feminized.

(9) In the studies of estrogen-like activity in the STP effluent, we concluded that estrogen, nonylphenols, and their derivatives remain dominantly in the final discharge. These compounds are likely transformed to other compounds and are also formed from their precursors. For more comprehensive evaluation of endocrine disrupting chemicals (especially, their fate and effects) in the STP streams and their receiving waters, a more accurate method need to be developed.

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