An Assessment of Endocrine Disrupting Activity Changes during Wastewater Treatment through the Use of Bioassays and Chemical Measurements

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ABSTRACT: The objective of this study was to assess the removal efficiencies of secondary wastewater treatment processes for compounds causing endocrine disrupting activity. The study used bioassays and chemical measurements, such as gas chromatography with mass spectrometry and enzyme immunosorbent assays. A total of seven full-scale water reclamation facilities using different unit operations and two pilot-scale membrane bioreactors were examined. Findings of this study imply that estrogenic disrupting activity in primary effluent is mainly caused by two steroidal hormones (17β-estradiol and estriol) and, to a lesser extent, by synthetic chemicals, such as bisphenol A, 4-nonylphenol, and 4-tert-octylphenol. During secondary treatment, steroidal hormones were removed to a higher degree than nonylphenol and bisphenol A. The total estrogenic activity was removed by an average of 96%. The remaining concentrations of targeted steroids in secondary effluents, except for estriol, still had the potential to elicit a positive response in the human breast cell cancer assay. For the majority of facilities, the remaining activity was likely attributed to residual concentrations of two steroidal hormones (17β-estradiol and estriol). *Water Environ. Res.,* **77,** 12 (2005).

KEYWORDS: bioassays, emerging contaminants, endocrine disrupting compounds, primary effluent, secondary treatment, steroidal hormones, water reclamation and reuse.

Introduction

An increasing number of utilities are recycling treated wastewater for beneficial reuse in nonpotable and potable applications. Results from recent research indicate that municipal wastewater effluents may contain compounds capable of endocrine disruption (Folmar et al., 1996; Harries et al., 1996; Purdom et al., 1994; Rodgers-Gray et al., 2000). Endocrine-disrupting compounds (EDCs) can be divided into three major classes according to their endpoints: (1) estrogenic compounds that mimic or block natural estrogens, (2) androgenic compounds that mimic or block natural testosterone, and (3) thyroidal compounds that cause direct or indirect effects to the thyroidal glands.

More than 70 000 chemicals are believed to have endocrine-disruptive potential (Bradley and Zacharewski, 1998). Hormonally active agents (e.g., 17β-estradiol, estrone, estriol, testosterone, and the synthetic hormone 17α-ethynylestradiol in contraceptives) exhibit a high endocrine-disrupting potency for various endpoints (Snyder et al., 2001). Some other chemical groups that can cause endocrine disruption and have been reported to occur in wastewater effluent include alkylphenolpolyethoxylates and their degradation products, bisphenol A, and some phthalates (Jobling and Sumpter, 1993; Montgomery-Brown et al., 2003). The endocrine-disrupting potency of the above compounds is appreciably lower compared to steroidal hormones (Fang et al., 2000). Nevertheless, because of their prevalence in domestic wastewater, EDCs might survive treatment during water reclamation, resulting in residual concentrations in receiving waters that have the potential of causing adverse effects to human health or aquatic life (Hemming et al., 2003). It is important to note that steroidal hormones, especially 17α-ethynylestradiol and 17β-estradiol, are often present in wastewater effluents at concentrations above the levels that are shown to cause adverse endocrine effects in fish (Baronti et al., 2000; Belfroid et al., 1999). However, because of the difficulty of measuring trace chemicals in wastewater effluent, data are often viewed skeptically because different analytical methods were used and method detection limits can vary by an order of magnitude (Huang and Sedlak, 2001; Snyder et al., 1999; Ternes et al., 1999). Further, mechanisms responsible for the attenuation of compounds causing endocrine disruption during wastewater treatment or water reclamation are not well understood. Conventional wastewater treatment facilities are not specifically designed to remove EDCs, and the degree with which they are removed vary from nearly complete to very little (Baronti et al., 2000; Holbrook et al., 2002; Ternes et al., 1999). There is a lack of understanding whether observed variations of target compound concentrations and activities are a result of the efficiency of a single-unit process, operational parameters (such as organic carbon loading), hydraulic retention time (HRT) and solid retention time (SRT), or sequences of unit processes used during water reclamation.

The results of multiple studies show that adsorption onto suspended solids, aerobic and anaerobic biodegradation, chemical (abiotic) degradation (via processes such as hydrolysis), and volatilization are the primary removal mechanisms for EDCs in wastewater treatment processes (Birkett and Lester, 2003; Holbrook et al., 2002; Mansell et al., 2004). Photolytic decay has also been suggested as a removal mechanism for steroidal hormones during exposure times exceeding 24 hours (Gray and Sedlak, 2003; Mansell et al., 2004). Many studies have suggested that increased SRTs improve the removal of EDCs in wastewater treatment plants (Andersen et al., 2003; Holbrook et al., 2002). Hydraulic retention time has also been reported to have a positive correlation with EDC removal (Strenn et al., 2003). Finally, there is a need to study removal, modification, or inactivation of EDCs and their metabolites using multiple techniques (including bioassays) to confirm chemical measurements and to assure that chemical concentrations are linked

Table 1—Target compounds, analytical methods, and 17β-estradiol potency factors.

Compound (CAS No.)	Analytical method	Detection limit (ng/L)	E-screen EC_{50} (M)*	Potency factor	EC_{50}* concentration (ng/L)
17α-Ethynylestradiol (57-63-6)	GC-NCI-MS	0.15	6.1×10^{-12}	1.36	1.79
17β-Estradiol (50-28-2)	GC-NCI-MS	0.15	8.9×10^{-12}	1.0	2.43
	HPLC-ELISA	0.4			
Estriol (50-27-1)	GC-NCI-MS	0.15	2.8×10^{-11}	0.297	8.19
	HPLC-ELISA	0.6			
Estrone (53-16-7)	GC-NCI-MS	0.15	6.7×10^{-11}	0.135	18.0
Testosterone (58-22-0)	GC-NCI-MS	0.15	6.4×10^{-7}	1.32×10^{-5}	184 864
	HPLC-ELISA	0.5			
4-tert-Octylphenol (140-66-9)	GC-NCI-MS	15	1.2×10^{-7}	9.74×10^{-5}	24 966
4-Octylphenol (1806-26-4)	GC-NCI-MS	15	1.6×10^{-6}	7.23×10^{-6}	336 318
Bisphenol A (80-05-7)	GC-NCI-MS	15	1.3×10^{-7}	7.95×10^{-5}	30 591
4-Nonylphenol (104-40-5)	GC-NCI-MS	15	1.9×10^{-7}	5.81×10^{-5}	41 867

* Note: EC_{50} = effective concentration.

to biological responses. Given the above, the scope of this study was to combine bioassays and chemical measurements to assess the efficacy of water reclamation processes, with special focus on secondary treatment, in removing estrogenic disrupting activity and androgens (testosterone) from treated effluents. During the overall study, five different bioassays were used (yeast estrogen assay, yeast androgen assay, human breast cell cancer assays [for estrogenic and androgenic endpoints—E-screen and A-screen], and the frog assay for a thyroidal endpoint). Results presented here, however, only report on E-screen results.

Methodology

Chemicals and Materials. All chemicals used were of reagent grade or higher, purchased from J. T. Baker, Inc. (Philippsburg, New Jersey), Sigma-Aldrich (St. Louis, Missouri), Eastman Organic Chemicals (Rochester, New York), and Fisher Scientific (Fairlawn, New Jersey). Reagent water (type I) was obtained from an ultrapure laboratory water purification system (Barnstead, Dubuque, Iowa). The average dissolved organic carbon concentration for the type I water was 0.06 mg/L.

Target Compounds. Target compounds selected for this study included steroidal hormones (17β-estradiol, estriol, 17α-ethynylestradiol, and testosterone), the main metabolite of 17β-estradiol (estrone), major degradation products of non-ionic surfactants (4-nonylphenol, 4-octylphenol, and 4-tert-octylphenol), and bisphenol A. These target compounds are characterized as hydrophobic, with log K_{ow} (octanol-water distribution coefficient) above 2. Endocrine-disrupting compounds in samples collected from various sites were quantified using enzyme-linked immunosorbent assays (ELISA), gas chromatography with mass spectroscopy (GC-MS), and the human breast cell cancer assay MCF-7 (E-screen). Target compounds, analytical methods used, and detection limits are summarized in Table 1.

Field Studies. Field investigations reported here focused on seven full-scale water reclamation facilities located in California, Arizona, Florida, Virginia, and Wisconsin, and two pilot-scale membrane bioreactor (MBR) facilities. This allowed for an investigation of both regional variations of water quality and the removal effectiveness of individual unit operations comprising an overall process train. Before sampling, staff of each facility provided detailed information on their service-area characteristics, treatment process, operational parameters, and water-quality data for the past four years. Technical details and operational parameters of each facility are summarized in Table 2. Full- and pilot-scale facilities were sampled at various locations throughout the treatment process, but this study only reports findings related to the secondary treatment process. Flow schematics of the secondary treatment process for each full-scale facility are illustrated in Figure 1. All full-scale facilities use activated-sludge treatment, and some use partial nitrification and nitrification–denitrification and chemical or biological phosphorus removal. Hydraulic retention times of the full-scale secondary treatment processes varied between 1.2 to 13.7 hours, with SRTs ranging from 1.7 to 10 days. The pilot-scale MBRs (provided by Kubota [Osaka, Japan] and Zenon Environmental [Oakville, Ontario, Canada]) used HRTs of 5 and 3 hours and SRTs of 10 and 21 days, respectively. The Zenon pilot-scale system was fed with primary effluent to a series of two anoxic and one aerobic tank, followed by a membrane tank containing 10 Zenon hollow fiber membrane elements. The nominal pore size of the membrane was 0.04 μm. The system was operated at a flux of 20 $L/m^2 \cdot h$ during sampling. The same primary effluent was fed to the Kubota MBR pilot-scale system consisting of one bioreactor equipped with two membrane units. Each membrane unit contained 50 flat plate cartridges composed of fine porous membranes. The average pore size of the Kubota membrane is 0.4 μm. During sampling, the system was operated at a flux of 51 $L/m^2 \cdot h$.

Sample Collection, Preparation, and Storage. Sampling at full-scale facilities occurred during dry weather, at flow conditions representing 56 to 100% of the design flows. Flow-proportional, hydraulically corresponding 4-hour composite samples were taken, after primary and after secondary clarification, at the clarifier weirs (sampling locations are indicated in Figure 1). At each sampling location, 6 liters of sample were collected, the pH was lowered to 2 to 3, and split samples were shipped overnight to the Wisconsin State Laboratory of Hygiene (Madison, Wisconsin) and the Colorado School of Mines (Golden, Colorado) in ice-packed coolers. Upon arrival, samples were filtered using a 0.45-μm cellulose acetate filter (Whatman, Maidstone, Kent, United Kingdom) and stored at 4°C. Within two days, samples were extracted, and analysis of extracts using E-screen, GC-MS, and high-pressure liquid chromatography (HPLC)-ELISA analyses was completed within two weeks. Each set of sample bottles shipped to a facility contained a bottle of high-purity water (field blank). In addition, for each batch of samples, both laboratories examined a high-purity water sample collected at

Table 2—Operational parameters for water reclamation facilites.

Facility	Wastewater treatment	Type of water reclamation and reuse	Design flow (m^3/d)	Sampling flow (m^3/d)	Hydraulic retention time (hours)	Solids retention time (days)
A	Nitrification, biophosphorus removal, UV disinfection	Nonpotable reuse: Wildlife habitat maintenance	94 636	76 900	13.7	10.0
B	Nitrification, chemical phosphorus removal, chlorination	Nonpotable reuse: Wildlife habitat maintenance	105 992	83 279	5.5	8.5
C	Preaeration, activated sludge, chlorination	Nonpotable reuse: Wildlife habitat maintenance	136 275	104 894	1.2	1.7
D	Nitrification, denitrification, tertiary filtration, chlorination (California Code of Regulations, Title 22), dechlorination	Nonpotable reuse: Landscape and agricultural irrigation	236 588	237 270	2.3	10.0
E	Nitrification, denitrification, tertiary filtration, UV disinfection	Indirect potable reuse: Surface spreading	68 137	37 854	5.0	10.0
F	Partial nitrification tertiary filtration, chlorination	Nonpotable reuse: Landscape and agricultural irrigation	87 064	68 932	6.8	4.4
G	Nitrification, denitrification, tertiary filtration, chlorination (California Code of Regulations, Title 22), dechlorination	Landscape and agricultural irrigation Industrial process water: wildlife habitat maintenance	238 481	238 481	7.0	7.0
H-1	Membrane bioreactor pilot plant (Kubota, Osaka, Japan)	Landscape irrigation	136	98	5.0	10.0
H-2	Membrane bioreactor pilot plant (Zenon Environmental, Oakville, Ontario, Canada)	Landscape irrigation	218	218	3.0	21.0

Note: Water quality observed during sampling campaign.

their laboratories (laboratory blank). Field and laboratory blanks were processed like field samples.

Analytical Methods

Biochemical Oxygen Demand and Nutrients. The biochemical oxygen demand (BOD) was determined through incubation for 5 days at 20°C, following Standard Method 5210B (APHA, et al., 1998). Kjeldahl nitrogen, ammonia, and nitrate-nitrogen were determined following Standard Methods 4500-N_{org} C, 4500-NH_3 F, and 4500-NO_3^- B, respectively. Operating and laboratory staff of participating facilities determined nutrient concentrations from split samples shared with the researcher laboratories, whereas BOD concentrations and operational parameters were based on weekly and daily averages, respectively.

Extraction for Gas Chromatography with Mass Spectroscopy and E-screen. Sample concentration was achieved by filtering a 1-L sample through a series of two high performance extraction disks (C18 and a polystyrenedivinylbenzene [SDB]-XC disk 3M Empore, St. Paul, Minnesota). The C18 disk was cleaned with 10 mL of a 50/50 dichloromethane (DCM)/ethyl acetate solution and conditioned with 10 mL methanol, followed by a 20-mL type I water rinse. The 1-L sample was filtered through the disk and then eluted first with 5 mL of ethyl acetate, then 5 mL of a 50/50 mixture of ethyl acetate and DCM, followed by 5 mL DCM only. The SDB disk was swelled and prewashed with acetone, followed by isopropyl alcohol. The disk was then washed with 10 mL of DCM. Finally, the disk was conditioned with 10 mL of methanol, followed by a 20-mL type I water rinse. The sample previously run through the C18 disk was modified by adding 50 g muffled sodium chloride, and the pH was lowered to 2 with DCM-cleaned hydrochloric acid. The sample was pulled through the disk, and the disk was eluted with three 5-mL rinses of DCM. The extracts from each disk were combined and blown to near dryness with nitrogen. The extracts were transferred with several ethanol rinses to a calibrated 2-mL amber vial, dried to near dryness, and brought back up to 1.5 mL in ethanol. Samples were stored in a 4°C cooler until use in the bioassay or GC-MS analysis.

Gas Chromatography with Mass Spectrometry. The ethanol extracts were analyzed by two different gas chromatography (GC)-negative chemical ionization (NCI)-mass spectrometry (MS) methods to quantify the hormones and phenolic compounds listed in Table 1. The 17β-estradiol, estrone, 17α-ethynylestradiol, and the phenols (4-nonylphenol, 4-tert-octylphenol, 4-octylphenol, and bisphenol A) were derivatized with pentafluorobenzoyl chloride (PFBCl) and analyzed by GC-NCI-MS (Kuch and Ballschmiter, 2001). Testosterone, estriol, 17β-estradiol, estrone and 17α-ethynylestradiol, and the phenols were derivatized with heptafluoric butyric acid anhydride (HFBAA) and analyzed by GC-NCI-MS. The primary quantification for testosterone and estriol was based on the HFBAA derivatives. The primary quantification for the remaining chemicals was based on the PFBCl derivatives. Detection limits for the target compounds were typically limited by field blanks and laboratory blanks but were typically in the range of 0.15 to 1.5 ng/L of water for the GC-NCI-MS analysis of the hormones. Results (mean ± standard deviation in ng/L) for the field blanks for the phenols were 2.92 ± 1.64 for nonylphenol, 0.66 ± 0.31 for 4-t-octylphenol, 10 ± 8 for 4-octylphenol, and 3.72 ± 2.22 for bisphenol A.

Table 2—Extended.

Mixed-liquor suspended solid (mg/L)	Organics loading biochemical oxygen demand (kg/day)	Influent biochemical oxygen demand* (mg/L)	Effluent biochemical oxygen demand* (mg/L)	Influent ammonia-nitrogen (NH_3-N*) (mg/L)	Effluent ammonia-nitrogen (NH_3-N*) (mg/L)	Effluent nitrate-nitrogen (NO_3-N*) (mg/L)
1900	34 913	227	4.1	23.5	0.02	13.1
2320	12 492	150	7	41	1.5	Not available
2180	18 042	172	4	30	33	<0.20
3660	40 810	260	2	34.5	2.4	6.64
2000	7041	186	4	25.5	0.13	6.59
2229	9857	143	4	28.5	4.75	2.05
2000	62 005	172	2.2	Not available	0.46	5.26
3660	16.9	172	2	36.6	15.5	6
3660	37.5	172	2	37.6	1	5.58

The 17β-estradiol-d_5 and bisphenol A-d_{16} were used as internal standards for the quantification of the hormones and phenolic compounds, respectively. The sample aliquots used for hormone analyses were spiked with both of the internal standards before derivatization. The PFBCl derivatization was performed by evaporating the sample to dryness using high-purity nitrogen gas and then resolvating the sample in 2 mL of high-purity water. Fifty μL of 2M potassium hydroxide (KOH) and 10 μL of 10% solution of PFBCl in toluene were then added to the resolvated sample. The derivative was extracted from the aqueous solution with hexane. The hexane extract was then analyzed by GC-NCI-MS, using methane as a reagent gas. The internal standard areas for each sample and standard were used to verify that the derivatization reaction was successful. Each batch of samples contained field blanks, laboratory blanks, and spiked samples for quality assurance and quality control purposes. The hormones, bisphenol A, 4-tert-octylphenol, and 4-octylphenol were quantified using multipoint calibrations curves generated with dilution of authentic standards. Nonylphenol was quantified using multipoint calibration curves generated with a mixture of nonylphenols. The quantification of nonylphenol was based on an average response factor for the numerous isomers that elute at similar retention times.

The sample aliquots for HFBAA derivatization were also blown to dryness using high-purity nitrogen after being spiked with the two internal standards. The sample was then resolvated with 100 μL of acetonitrile and 120 μL of HFBAA. After vortexing for 20 seconds, the mixture was heated for 1 hour to 60°C. The derivative reaction mixture was then reevaporated to dryness using high-purity nitrogen. The derivatized products were then solvated with 1 mL of *n*-hexane and then analyzed by GC-NCI-MS under the same conditions as the PFBCl derivatives.

High-Pressure Liquid Chromatography–Enzyme-Linked Immunosorbent Assays. The analytical method used for quantification of steroids HPLC-ELISA in water samples is described in detail in Mansell et al. (2004). Samples were processed through a C-18 solid phase extraction disk (Empore, St. Paul, Minnesota). Organic matter was extracted from the C-18 disks using methanol–water solutions. The extracts were dried, resuspended in acetonitrile–water solutions, and subjected to two HPLC fractionation steps using a size-exclusion and a reversed phase column (Alltech, Deerfield, Illinois), which were used to isolate the target compounds from background organic matter. Quantification of the steroids in the samples was performed using ELISA. The 17β-estradiol, estriol, and testosterone ELISA kits were obtained from Cayman Chemicals (Lansing, Minnesota). The recoveries for all three target compounds through the extraction procedure and the HPLC cleanup were 95 ± 3%. Recoveries ranged from 45 to 95% when isolated compounds where quantified using ELISA. Recoveries of all compounds were verified using a standard addition of 30 ng/L added to a split sample before solid-phase extraction. Laboratory blank samples using ultrapure water were processed with each batch of field or laboratory samples. The detection limit of the analytical method for a 1-L sample for 17β-estradiol, estriol, and testosterone was calculated according to Standard Methods (APHA et al., 1998), by considering three times the standard deviation of the blank samples. Limits of detection for 17β-estradiol, estriol, and testosterone were 0.4, 0.6, and 0.5 ng/L for a 1-L sample, respectively.

E-Screen. A breast cancer cell line (MCF-7 cells) was obtained from Drs. Sonnenschein and Soto at Tufts University (Boston, Massachusetts) for use in the E-screen. The MCF-7 cells were cultured in Dulbecco's modified eagle's medium (ICN Biomedicals, Aurora, Ohio), with 5% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah) at 37°C and 6.5% carbon dioxide in 75 cm^2 tissue culture flasks. Media was changed every 2 to 3 days, and the cells were subcultured every seven days. Stocks of MCF-7 cells were stored in liquid nitrogen, and new cells were thawed to replace cells that have undergone approximately 25 passages.

The methods for the E-screen were based on those described by Soto et al. (2004). The MCF-7 cells were seeded in 24-well plates to achieve 20 000 to 30 000 cells per well. Twenty-four hours after seeding, the media was removed and experimental media (charcoal dextran [CD] media) was added. The experimental media was Dulbecco's modification of eagle's medium without phenol red (Irvine Scientific, Irvine, California), while the 5% FBS used in this media was stripped of steroids with a CD stripping procedure. Each set of experiments was conducted with a 17β-estradiol standard curve consisting of 15 concentrations of 17β-estradiol (Sigma-Aldrich, St. Louis, Missouri) in CD-media ranging from 0.027 to 2724 ng/L. All treatments were done in quadruplicate, with three plates needed to obtain a complete standard curve. Four control wells were included on each plate.

To determine the estrogenic activity of the samples, the sample extract (in ethanol) was added to CD-media at a concentration no greater than 1%, and a dilution series was made to ensure that active samples fell within the linear portion of the standard curve. Each sample dilution was applied to four wells at a volume of 0.5 mLs. In one of those wells, 17β-estradiol was added to obtain a concentration of 27.4 ng/L in the well. This positive control was used to determine whether anything in the sample was preventing the growth of the cells. Again, each plate contained four control wells.

To determine the relative potency of the target chemicals (17β-estradiol, estrone and 17α-ethinylestradiol, estriol, estrone, testosterone, 4-nonylphenol, 4-tert-octylphenol, 4-octylphenol, and bisphenol A) using the E-screen, dose-response curves for each of the compounds were made using at least eight different concentrations. Each treatment was done in quadruplicate, and four control wells were included on each plate. Each chemical was tested in at least two independent runs.

After five days of incubation, cell proliferation was measured by the sulphorhodamine B (SRB) (Sigma-Aldrich, St. Louis, Missouri) protein assay. The SRB assay determines total cell numbers by measuring total protein content. The plates were read at a wavelength of 515 nm on a microplate reader (Molecular Devices, Sunnyvale, California). The standard curve was fit with a four-parameter logistic equation with Softmax PRO v. 2.6 (Molecular Devices, Sunnyvale, California) software package, the software analysis package for the microplate reader. To determine the estradiol equivalents (EEQ) of the samples, results were interpolated from the standard curve and corrected for the dilution and concentration of the samples. The limit of detection was 0.04 ng/L.

The concentration causing 50% of the maximum cell proliferation (EC_{50}) for the dose-response curves of the target compounds was calculated using Microcal Origin v. 4.1 (Microcal, Northampton, Massachusetts). The potency for each chemical was determined relative to the EC_{50} of 17β-estradiol (Table 1). The estrogenic activity that was expected, based on the chemical analyses, was determined by multiplying the concentration of the chemical found by the potency factor. The expected EEQ for each sample was calculated as the sum of the activities for the analyzed chemicals. With the exception of 4-octylphenol, all of the target chemicals exhibited similar levels of maximum proliferation. The maximum proliferation for 4-octylphenol was only approximately 20% of the maximum for the other chemicals.

Results

Baseline Performance of Investigated Facilities during Sampling. All facilities investigated in this study received untreated domestic wastewater from urban settings located in different areas of the United States. The design capacity of the full-scale facilities varied between 68 000 and 238 500 m^3/day. The Kubota and Zenon MBR pilot facilities were operated at a flow rate of 98 and 218 m^3/d, respectively. The untreated wastewater at all sites was characterized as medium-strength wastewater based on five-day BOD (BOD_5), ammonia-nitrogen, and phosphorus concentrations (Metcalf and Eddy, 2003). During the sampling campaigns, all facilities achieved a BOD removal exceeding 95% and nitrogen removal similar to values determined by the initial utility survey, indicating performance within design specifications (Table 2).

Occurrence of Endocrine-Disrupting Compounds in Primary and Secondary Effluents. The concentrations of target compounds observed in the primary and secondary effluents are summarized in Table 3. Concentrations of individual target compounds were quantified using GC-NCI-MS and HPLC-ELISA. The 17β-estradiol and testosterone concentrations, which were determined with both analytical methods, showed a good agreement for secondary effluent samples and for most of the primary effluent samples. Primary effluent samples for estriol, however, exhibited significant differences between the two methods, which might be attributed to organic-matter matrix effects that occur while processing primary treated wastewater samples. While both analytical methods had similar detection limits for 17β-estradiol (<0.6 ng/L), the HPLC-ELISA method for estriol and testosterone showed a better sensitivity at the low concentration range, with a detection limit below 1 ng/L for both compounds.

The 17α-ethynylestradiol was only detected in four out of eight primary effluents, at a concentration range varying between 1.9 and 14.4 ng/L. Only one secondary effluent sample, collected at facility C, exhibited 17α-ethynylestradiol concentrations above the detection limit of 0.7 ng/L. Facility C is the plant with the shortest HRT and SRT from all facilities investigated. Concentrations for 17α-ethynylestradiol below 1 ng/L in treated wastewater are in agreement with findings from other studies reporting similar median concentrations for secondary treated effluents (Belfroid et al., 1999; Desbrow et al., 1998; Huang and Sedlak, 2001; Snyder et al., 1999). 17β-estradiol, estrone, and estriol were prevalent in every primary effluent in concentrations exceeding 7 ng/L, 26 ng/L, and 138 ng/L, respectively, based on GC-NCI-MS measurements. Estrone, the metabolite of 17β-estradiol, exhibited the highest concentration of all steroidal hormones in secondary treated effluents. Testosterone consistently showed concentrations exceeding 19 ng/L, based on GC-NCI-MS measurements in primary effluents. Secondary treatment removed testosterone to concentrations below 6 ng/L, based on HPLC-ELISA measurements, with four facilities exhibiting concentrations less than 0.5 ng/L in their treated effluent. Similar testosterone concentrations for primary and secondary effluents have been reported by Svenson and Allard (2004). Although present in the primary effluent, 17α-ethynylestradiol, 17β-estradiol, estriol, and testosterone were not detected in both MBR effluents. Only estrone was detected in the Kubota MBR effluent at a concentration of

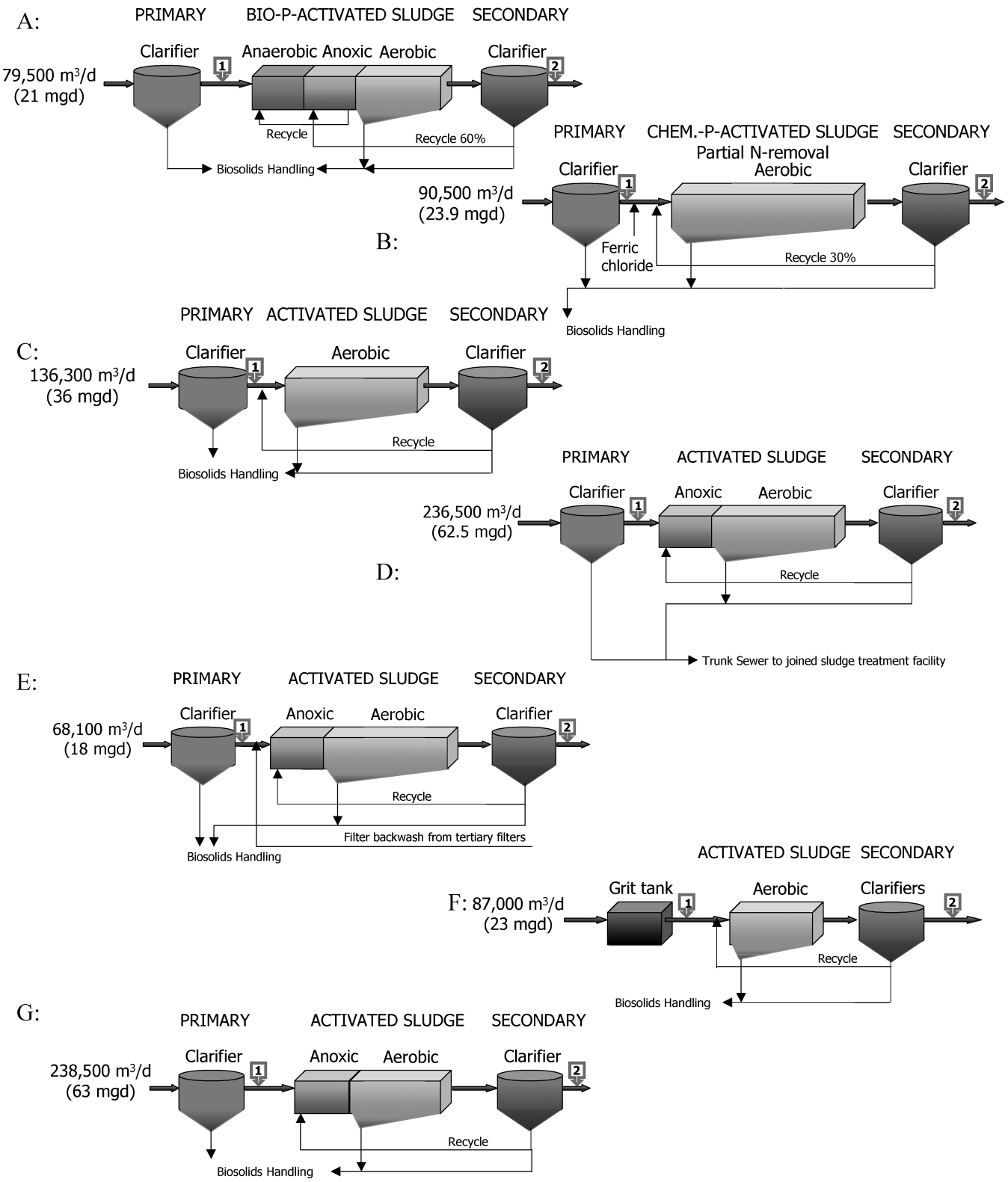

Figure 1—Primary and secondary treatment processes of investigated full-scale facilities.

5.5 ng/L, while estrone was below the detection limit in the Zenon MBR effluent.

Concentrations of 4-tert-octylphenol and 4-nonylphenol in primary effluents exhibited the largest variations representing differences of two orders of magnitude. Elevated concentrations of 4-nonylphenol in primary effluent samples were generally associated with elevated concentrations of 4-tert-octylphenol. The 4-octylphenol was below the method quantification limit of 124 ng/L in all

Table 3—Occurrence of target compounds in primary and secondary treated effluents in nanograms per liter.

			17β-estradiol equivalents										
			17α-ethynyl-estradiol	17β-estradiol		Estriol		Estrone	Testosterone		4-tert-Octylphenol	Bisphenol A	4-Nonyl phenol
Facility	Method	E-screen	GC-NCI-MS	GC-NCI-MS	HPLC-ELISA	GC-NCI-MS	HPLC-ELISA	GC-NCI-MS	GC-NCI-MS	HPLC-ELISA	GC-NCI-MS	GC-NCI-MS	GC-NCI-MS
A	Primary effluent	17.4	1.9	7.0	19.9	261.4	15.9	41	75.2	135.8	>16 704	3642	>8172
	Secondary effluent	0.21	<0.7	<1	6.2	<2	<0.4	0.6	<1	6.2	Not applicable	Not applicable	1428
B	Primary effluent	59.3	9.1	8.8	37.5	307	19.2	42	27	35.1	>16 704	Not applicable	>8172
	Secondary effluent	1.55	<0.7	<1	<0.6	<1	<0.4	<1	<1	1.3	Not applicable	Not applicable	4926
C	Primary effluent	42	14.4	24.5	4.6	138	15.8	60	19.4	80.8	2140	470	13 080
	Secondary effluent	7.91	4.1	4.4	<0.6	4.0	<0.4	17.7	<3.0	1.9	49	19	2177
D	Primary effluent	94.7	<0.7	23.9	37.6	147	29.9	60.3	21.7	79.1	443	281	1840
	Secondary effluent	3.12	<0.7	1.5	<0.6	<4.7	<0.4	50.4	<4.1	<0.5	68	14	501
E	Primary effluent	94.1	<0.7	15.3	N/A	381	38.3	65.2	43.6	49.1	1250	328	16 000
	Secondary effluent	1.57	<0.7	6	4.1	4.9	0.7	11.1	<2.9	4.1	107	14	1775
F	Primary effluent	35.1	<0.7	12	25.9	150	2.7	39.4	24.5	45.2	1650	530	1800
	Secondary effluent	0.18	<0.7	<0.6	0.69	<3.3	<0.4	27.5	<2.9	<0.5	180	50	1200
G	Primary effluent	32.9	<0.7	12.8	13.1	168	<0.4	80.3	57	188	2000	323	27 411
	Secondary effluent	2.1	<0.7	1.8	0.62	<3.3	<0.4	16.4	4.9	2.4	53	28	1560
H-1	Primary effluent	81.5	10.3	14.1	11.1	250	7.8	26.3	143	50.4	300	360	1570
	Secondary effluent	1.35	<0.7	<0.6	<0.6	3.9	<0.4	5.5	1.6	<0.5	135	11	640
H-2	Primary effluent	81.5	10.3	14.1	11.1	250	7.8	26.3	143	50.4	350	360	1570
	Secondary effluent	0.62	<0.7	<0.6	<0.6	<3.4	<0.4	<3.9	1.6	<0.5	37.5	6	426

primary and secondary treated effluent samples. Secondary effluent concentrations of 4-tert-octylphenol for all facilities were below 180 ng/L. Concentrations of 4-nonylphenol in secondary treated effluents were significantly lower than the primary effluent concentrations and rarely exceeded 2100 ng/L. The concentration range observed in secondary effluent samples is consistent with findings from other studies (Johnson and Sumpter, 2001; Snyder et al., 1999). Bisphenol A rarely exhibited concentrations exceeding 530 ng/L in primary effluents. The facility where the highest concentration of bisphenol A was observed also showed a high 4-nonylphenol concentration and the highest concentration of 4-tert-octylphenol of all sites investigated.

The total estrogenic activity quantified through the E-screen varied in primary effluents between 17 and 95 ng/L. For nitrifying–denitrifying facilities, the 17β-estradiol equivalent concentration in secondary treated effluents varied between 0.18 and 3.12 ng/L. The highest E-screen concentration (7.9 ng/L) was determined in the secondary effluent of facility C, which exhibited the shortest SRT and does not nitrify. In general, the occurrence of EDCs did not show a regional pattern considering the different locations and climates of the various facilities.

Endocrine-Disrupting Activity and Occurrence of Endocrine-Disrupting Compounds. By considering endocrine-disrupting potency factors (Table 1), the presence of individual target compounds in effluent samples can be expressed in 17β-estradiol equivalent concentrations. The sum of target compounds expressed in EEQ concentrations can be compared to the biological response in the E-screen as shown in Figure 2. Given the low estrogenic potency of testosterone, the HPLC-ELISA results only consider 17β-estradiol and estriol equivalent concentrations, while the GC-NCI-MS results consider the equivalent concentrations of all hormones (except testosterone) and phenolic compounds. In general, the total

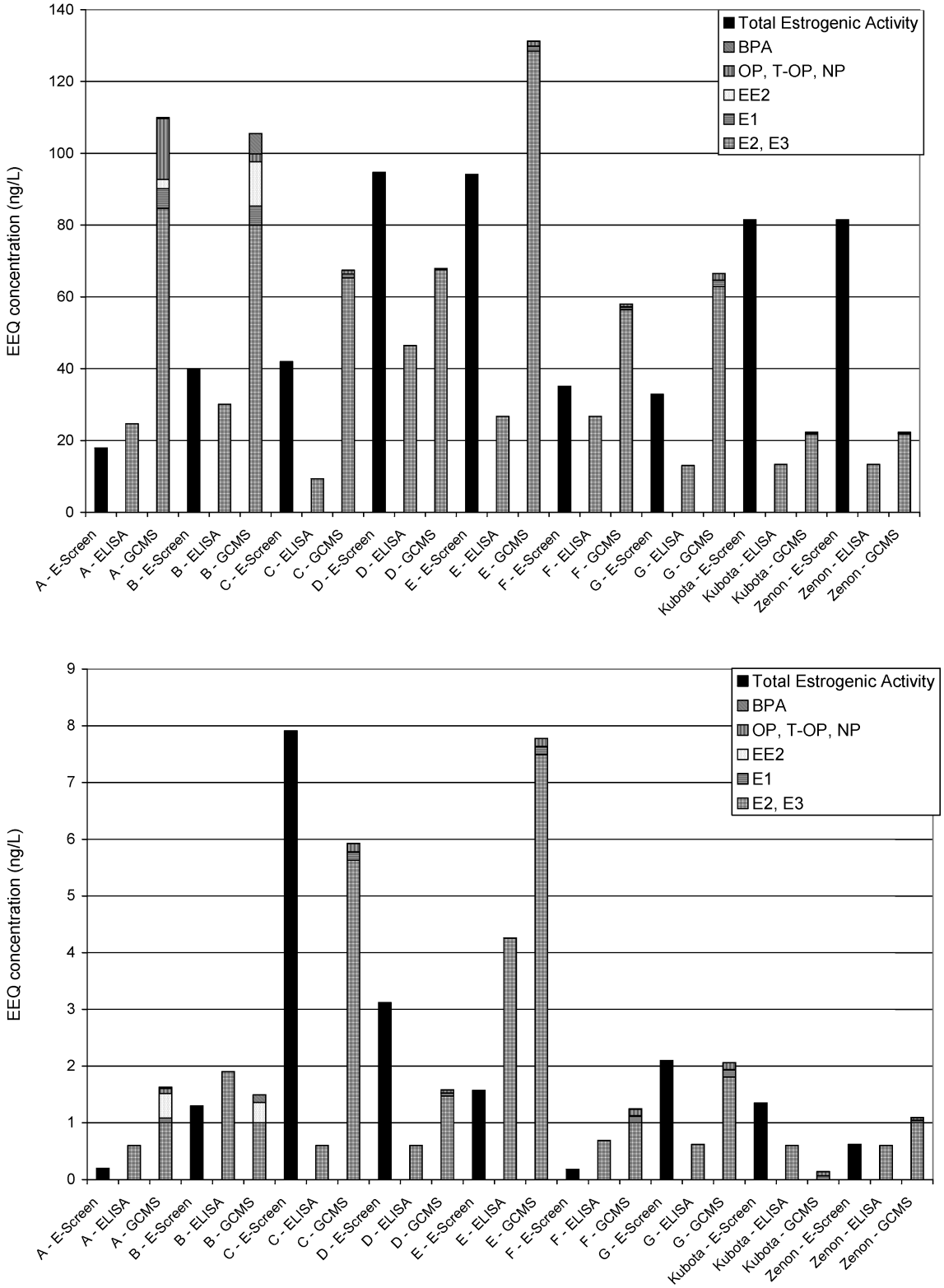

Figure 2—Estrogenic activity in primary and secondary effluents.

estrogenic activity in all primary effluents (except for facility D) was lower than the sum of individual target compounds based on GC-NCI-MS measurements. Findings suggest that the total estrogenic activity was mainly caused by 17β-estradiol and estriol, with rather small contributions from estrone, 17α-ethynylestradiol, and phenolic compounds. For secondary effluents, except for two facilities (C and D), chemical measurements could account for the total estrogenic activity and, similar to the observations in primary effluents, this

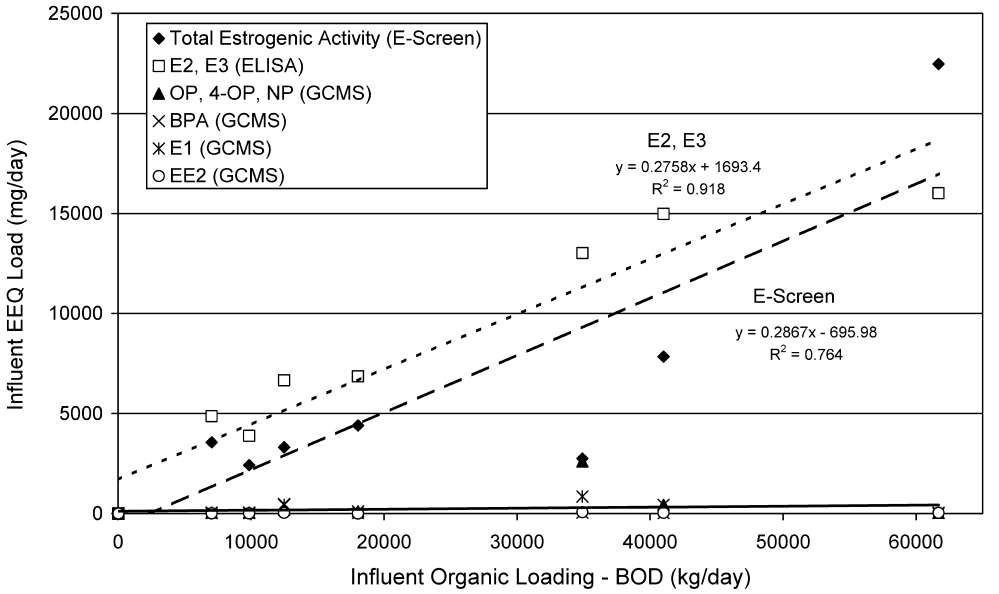

Figure 3—Influent organic loading (as BOD_5) vs. influent estradiol equivalent (EEQ) loading for all facilities investigated.

activity seemed to be mainly caused by the presence of 17β-estradiol and estriol.

Biochemical Oxygen Demand Loading and Presence of Endocrine-Disrupting Activity in Primary Effluent. The organic loading to a secondary treatment process is generally expressed in BOD mass delivered per time. By considering all treatment facilities, an increased influent organic loading resulted in an elevated influent estrogenic activity loading (Figure 3). The influent estrogenic activity loading, determined as the total estrogenic activity exhibited in the E-screen, showed a positive correlation with the BOD loading to wastewater treatment plants (R^2 = 0.76). The 17β-estradiol and estriol exhibited an even stronger correlation (R^2 = 0.92) between the influent EEQ loading and an increased BOD loading. This contribution was slightly higher than the total estrogenic activity, confirming the dominant contribution of 17β-estradiol and estriol to the total activity in primary effluents.

Removal of Estrogenic Activity during Secondary Treatment. During secondary treatment, the total estrogenic activity was removed by 96%, on average (Figure 4). 17β-estradiol/estriol, 17α-ethynylestradiol, and testosterone exhibited average removal efficiencies of 98, 94, and 96% during secondary treatment. Estrone was only removed by 85%, which is most likely because of additional estrone contributions from 17β-estradiol because of an incomplete mineralization. Bisphenol A was removed by 90%.

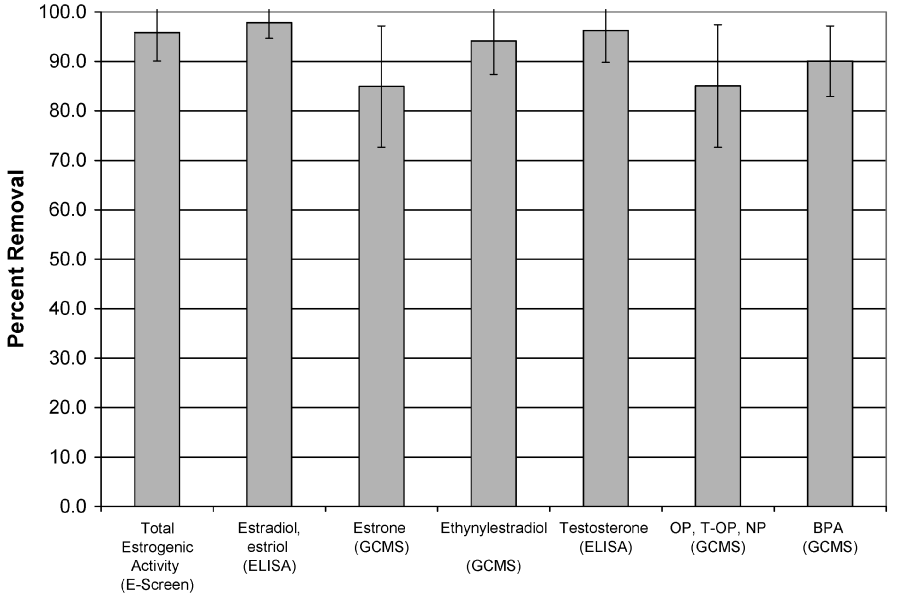

Figure 4—Average removal efficiencies and standard deviations of total estrogenic activity (based on E-screen) and individual endocrine-disrupting compounds during secondary treatment.

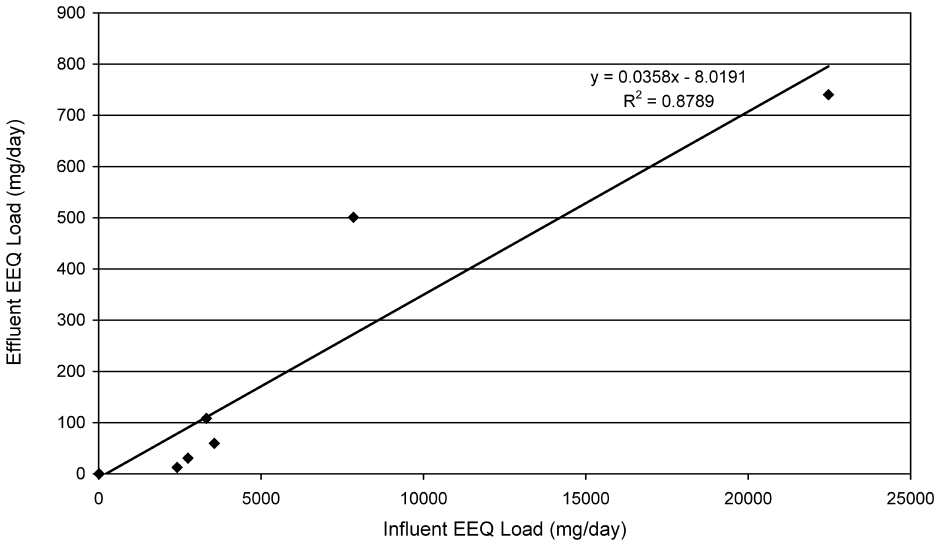

Figure 5—Secondary effluent total estrogenic loading vs. primary effluent total estrogenic loading for nitrifying–denitrifying facilities.

4-tert-octylphenol/4-octylphenol/4-nonylphenol exhibited a lower removal efficiency compared to the steroidal hormones and bisphenol A, with an average removal of 85%.

Discussion

17β-estradiol and estriol accounted for approximately 75 to 100% of the total estrogenic activity present in primary effluents, which varied between 17 and 95 ng/L, expressed as 17β-estradiol equivalents. 17α-ethynylestradiol, the most potent estrogenic-disrupting compound, was only detected in four out of eight primary effluents, at concentrations below 15 ng/L. 17β-estradiol, estriol, estrone, and testosterone were prevalent in almost all primary effluents in the low nanogram-per-liter range, with estriol exhibiting the highest concentration of all steroids. 4-tert-octylyphenol and 4-nonylphenol exhibited the largest variations in primary effluents, with concentrations in the low microgram-per-liter range. Bisphenol A typically exhibited concentrations exceeding 300 ng/L in primary effluents. However, 4-tert-octylphenol, nonylphenol, bisphenol A, and 17α-ethynylestradiol contributed very little to the total estrogenic activity present in primary effluents. The total estrogenic activity in primary effluent and 17β-estradiol and estriol showed a positive correlation, with the BOD loading to wastewater treatment plants, indicating that EDCs are generally occurring in similar concentrations in raw wastewater, independent of the location of a plant.

The total estrogenic activity removed during secondary treatment correlated well with BOD removal. These findings imply that, within a range of BOD mass removal between 16 and 61 000 kg/day, secondary treatment processes are not limited in their capability to remove estrogenic activity and their main contributors, 17β-estradiol and estriol. It can also be concluded that the removal of estrogenic activity follows mechanisms also responsible for BOD removal, namely biodegradation and adsorption to biosolids. Steroidal hormones, the main contributors to the total estrogenic activity, were removed by more than 94% during secondary treatment. Testosterone was removed by 96% in activated-sludge systems. The remaining concentrations for estriol in secondary effluents were below the concentration, with 50% effect in the E-screen (EC_{50}), whereas some of the concentrations of 17α-ethynylestradiol, 17β-estradiol, and estrone observed in secondary treated effluent still had the potential to cause an endocrine-disrupting effect in the human breast cell cancer assay. 4-tert-octylphenol and bisphenol A, occurring at significant higher concentrations, were removed during secondary treatment to concentrations three orders of magnitude below the 50% effect level of the E-screen. 4-nonylphenol was less efficiently removed during secondary treatment, exhibiting average removal efficiencies of 70%. The remaining concentrations of 4-nonylphenol in secondary effluents, however, were more than one order of magnitude below the EC_{50} of the E-screen. The two pilot-scale MBRs achieved the lowest concentrations of individual EDCs in secondary treated effluents. Their performance was better than the conventional full-scale facility D, which is operated in the same service area. The remaining total estrogenic activity in secondary effluents of all facilities was low, but above the detection limit of the E-screen. For the majority of facilities, the remaining activity is likely attributed to residual concentrations of target compounds, likely two steroidal hormones (17β-estradiol and estriol).

It is interesting to note that higher primary effluent 17β-estradiol equivalent concentrations (as determined by the E-screen) correlated well with higher secondary effluent equivalent concentrations (Figure 5), implying that secondary treatment processes cannot completely remove estrogenic-disrupting activity. It is also noteworthy that facilities with longer SRTs (exceeding 4.4 days) exhibited a lower total estrogenic activity than the facility operating at a very short SRT of 1.7 days, confirming observations reported by Lee et al. (2003) and Strenn et al. (2003). The same is true for HRTs, where a short retention time of only 1.2 hours resulted in the highest total estrogenic activity. Finally, no correlation existed between higher mixed-liquor suspended solid concentrations and an improved EDC removal.

Conclusions

Findings of this study imply that the occurrence of EDCs in primary effluent is coupled with the organic loading rate of a plant. Strong correlations exist between the BOD loading and influent loading of two hormones (17β-estradiol and estriol) and the total estrogenic activity. Secondary treatment processes were partly

efficient in removing both steroidal hormones and phenolic compounds, resulting in a reduction of total estrogenic activity of more than 94%. Testosterone concentrations were reduced by 96% during secondary treatment. However, secondary treatment alone cannot assure a complete removal of estrogenic-disrupting activity from wastewater. Based on findings of this study, removal of estrogenic-disrupting activity during secondary treatment is dependent on the total mass load of endocrine-disrupting activity to a plant, which strongly correlated with the BOD loading, and a SRT of at least four days. Findings indicate that the estrogenic activity in secondary effluents seems to be mainly derived from two steroidal hormones (17β-estradiol and estriol).

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