ANALYSIS OF ESTROGENIC HORMONES IN MUNICIPAL WASTEWATER EFFLUENT AND SURFACE WATER USING ENZYME-LINKED IMMUNOSORBENT ASSAY AND GAS CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

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Abstract—Although the estrogenic hormones 17β-estradiol and 17α-ethinyl estradiol can be quantified in polluted waters by gas chromatography/mass spectrometry (GC/MS) and gas chromatography/tandem mass spectrometry (GC/MS/MS), the compounds often are present at concentrations below detection limits. Enzyme-linked immunosorbent assays (ELISAs) provide a sensitive and robust means of quantifying estrogenic hormones in wastewater effluents and surface waters. Results from ELISA analysis of estrogenic hormones in secondary wastewater effluent indicate concentrations comparable to those that cause vitellogenesis in fish. Confirmatory analyses by GC/MS/MS are consistent with ELISA results. Effluent filtration, using sand filtration or microfiltration, removes approx. 70% of the hormones from secondary effluent, while advanced treatment, using reverse osmosis, removes more than 95% of hormones. The detection limits for estrogenic hormones are approx. 0.1 ng/L in wastewater effluent and 0.05 ng/L in surface water. The ELISA technique provides a relatively simple and practical method of assessing the fate of estrogenic hormones in engineered and natural systems.

Keywords—17β-estradiol   17α-ethinyl estradiol   Wastewater   Enzyme-linked immunosorbent assay   Endocrine disruption

INTRODUCTION

In recent years, endocrine disruption has been observed in wild fish and in fish caged in rivers that receive significant inputs of wastewater effluents [1–4]. As a result, concerns have been raised about the presence of endocrine-disrupting chemicals in wastewater effluents. Among the large number of chemicals potentially responsible for endocrine disruption in fish, natural and synthetic estrogenic hormones (e.g., 17β-estradiol and 17α-ethinyl estradiol) are the most likely candidates [5,6]. Humans produce and excrete relatively large quantities of estrogenic hormones. For example, between 0.002 and 0.10 mg of the endogenous hormone 17β-estradiol is excreted by humans each day. Pregnant women excrete as much as 30 mg/d of 17β-estradiol [7,8]. In addition, natural and synthetic estrogens are used in large quantities for medicinal purposes, such as oral contraceptives and hormone replacement therapy. Estrogenic hormones are excreted by mammals as sulfate or glucuronide conjugates in urine and in unmetabolized forms in feces [9]. Although the conjugated hormones are inactive, they can be converted back into their original forms with glucuronidase or sulfatase enzymes [9].

The possible presence of estrogenic hormones in wastewater was hypothesized by Stumm-Zollinger and Fair in 1965 [10]. However, analytical techniques capable of detecting the compounds at the concentrations expected in wastewater were unavailable. Later attempts to detect estrogenic hormones in wastewater effluents and in natural waters have employed solid-phase extraction followed by analysis using gas chromatography/mass spectrometry (GC/MS) [5,11,12]. Despite the sensitivity of these techniques, accurate quantification is difficult because a high concentration factor is required, and natural organic matter in the concentrated extracts can interfere with analysis. As a result, some analyses have yielded concentrations of hormones considerably greater than concentrations predicted in wastewater influents [13].

More recent attempts to quantify estrogenic hormones have employed gas chromatography coupled with tandem mass spectrometry (MS/MS). The GC/MS/MS is less susceptible to interference by natural organic matter than GC/MS [14,15]. However, the detection limits are still higher than the concentrations of hormones in many wastewater effluent and surface water samples. Concentrations of estrogenic hormones below GC/MS/MS detection limits are a concern because endocrine disruption has been reported in fish exposed to 17β-estradiol and 17α-ethinyl estradiol at concentrations as low as approx. 1 ng/L [1,6], and additive or synergistic effects could result in responses at even lower concentrations.

Immunoassays could provide an alternative approach for quantifying estrogenic hormones in the presence of natural organic matter. Immunoassays are simple, inexpensive, and extremely sensitive. Although immunoassays have been used for many environmental analyses [16], the complex matrices encountered in wastewater and surface water extracts present new challenges for this technique. The use of radioimmunoassays to quantify estrogenic hormones in aquatic environments was reported by Snyder et al. [17]. However, confirmatory analyses were not performed. Furthermore, radioimmunoassays are not commercially available, while enzyme-linked immunosorbent assay (ELISA) kits for estrogenic hormones are sold by several manufacturers.

This study was performed to assess the ability of commercially available ELISAs to quantify estrogenic hormones and their conjugates in wastewater effluents from municipal wastewater treatment plants and surface waters. The GC/MS/MS was used to confirm the results of ELISA. In addition, samples were

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0.3-2.0 L Wastewater
2.0-6.0 L Surface water

↓

solid phase extraction

enzymatic hydrolysis of
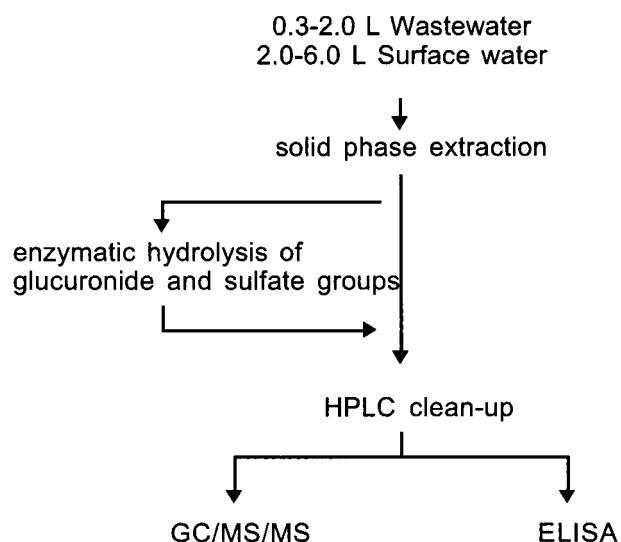glucuronide and sulfate groups

HPLC clean-up

GC/MS/MS   ELISA

Fig. 1. Summary of analytical procedure. HPLC = high-performance liquid chromatography, GC/MS/MS = gas chromatography/tandem mass spectrometry, ELISA = enzyme-linked immunosorbent assay.

collected from wastewater treatment plants using conventional and advanced treatment processes to assess the removal efficacy of different treatment technologies.

MATERIALS AND METHODS

Unless otherwise specified, all chemicals were obtained from Fisher Scientific (Pittsburgh, PA, USA) at the highest possible purity. Estrogenic hormones were purchased from Sigma (St. Louis, MO, USA). Aqueous solutions were prepared in deionized water produced by a Nanopure system (Barnsted, Dubuque, IA, USA). Stock solutions of 17β-estradiol and 17α-ethinyl estradiol were initially prepared in methanol at 53.2 mg/L and were subsequently diluted with deionized water to a concentration of 10 μg/L.

The process used to quantify estrogenic hormones involved a series of steps to concentrate the hormones, separate them from interfering compounds, and, for GC analysis, convert them into derivatives that were more easily detected. A schematic representation of the analytical technique is provided in Figure 1 and is described below.

Sample collection, extraction, and recovery experiments

Wastewater samples were collected after disinfection at four municipal wastewater treatment plants (WWTPs) located in California, USA. The characteristics of the wastewater treatment plants are listed in Table 1. Wastewater treatment plants 1 and 2 use activated sludge for secondary treatment and chlorination for disinfection. Wastewater treatment plant 2 also employs biological nutrient removal and sand filtration. Wastewater treatment plant 3 is an advanced wastewater treatment plant equipped with a water hyacinth pond for primary and secondary treatment followed by lime precipitation, sand filtration, membrane filtration, reverse osmosis, ozonation, and ultraviolet (UV) disinfection. Wastewater treatment plant 4 employs a trickling filter secondary treatment system followed by UV disinfection.

Surface water samples were collected from an engineered wetland that receives only secondary effluent from WWTP 4. Surface water also was sampled from the Colorado River, near the intake point for San Diego's water supply, and from the Sacramento River Delta near Patterson Pass.

All samples were collected and stored in fluorinated high-density polyethylene bottles. Wastewater effluent samples were filtered on site or immediately after being brought back to the laboratory using a peristaltic pump equipped with Teflon® tubing and a 0.22-μm polypropylene in-line filter cartridge (MSI, Westborough, MA, USA). Surface water samples were filtered immediately after being brought back to the laboratory using a stainless-steel pressurized filtration system with a 90-mm glass-fiber prefilter and 0.7-μm glass-fiber filter (Millipore, Bedford, MA, USA). To prevent adsorption of dissolved hormones onto the filters, filters were equilibrated by passing at least 100 ml of sample through the cartridge prior to collection of samples. Filtered samples were stored at 5°C until extraction and analysis. Most samples were extracted within 24 h of collection.

Estrogenic hormones were extracted from water using C-18 solid-phase extraction discs (Empore, 3M, Minneapolis, MN, USA). Prior to extraction, the discs were conditioned with methanol and water. Between 0.3 and 2 L of each wastewater effluent sample were extracted with 47-mm discs by applying vacuum to maintain a flow rate of 0.5 to 5 ml/min. Between 2 and 6 L of each surface water sample was extracted with 90-mm discs using a stainless-steel pressurized filtration system at a pressure less than 30 psi and a flow rate of 45 to 60 ml/min. Hormones were eluted from the C-18 discs with 15 to 30 ml of methanol, blown down to dryness under a gentle stream of nitrogen gas, and reconstituted in 600 μl of deionized water.

To evaluate the accuracy and precision of the method, three to four duplicates and recovery samples were analyzed with each batch of samples. Blank samples (deionized water) also were analyzed to ensure that contamination did not occur during the analytical procedures. Recoveries were evaluated by amending aliquots of samples with 3 to 10 ng/L of hormones or hormone conjugates prior to extraction. To calculate recoveries, concentrations of hormones and their conjugates measured in the same sample were subtracted from concentrations measured in the recovery samples.

Enzymatic hydrolysis

Aliquots (60–90 μl) of concentrated samples were subjected to enzymatic hydrolysis to convert glucuronide and sulfate conjugates into active hormones. Each hydrolysis experiment was performed in 4 ml of glucuronidase enzyme (Sigma, Type H-1 isolated from *Helix pomatia*) solution, which was prepared daily at a concentration of 1.9 g/L (equivalent to 800 units/ml) in 0.1 M acetic acid buffer adjusted to pH 5.0 with 0.1 M NaOH. Some experiments also were performed using type L-2 glucuronidase enzyme. Hydrolysis was allowed to proceed for 16 to 20 h at 35°C. The activity of the enzyme was confirmed in every experiment by hydrolyzing 90 μl of 3-μg/L 17β-estradiol-17-glucuronide stock solution in 4 ml glucuronidase enzyme solution. Concentrations of conjugated hormones were determined by differences between hydrolyzed and unhydrolyzed samples.

To evaluate the efficacy of the enzymatic hydrolysis process, kinetics experiments were conducted in distilled water amended with 800 units/ml of type H-1 and type L-2 glucuronidase enzyme. Results indicated that both enzymes quantitatively convert 17β-estradiol glucuronide into 17β-estradiol. However, the enzymes convert only approx. 30% of the 17β-estradiol sulfate into 17β-estradiol. This is consistent with previous studies in which incomplete conversion of sulfate conjugates into active hormones has been reported [18]. As a result, enzymatic hydrolysis may underestimate the concentration of sulfate-conjugated hormones.

Table 1. Characteristics of wastewater treatments plants sampled[a]

WWTP	Design flow MGD (m³/s)	Primary treatment	Secondary treatment	Tertiary treatment	Advanced treatment[b]	Disinfection
1	12 (0.50)	X	X[c]		X	HOCl
2	170 (7.3)	X	X[c]	X[d]		HOCl
3	0.50 (0.022)	X	X	X[e]	X	O₃ and UV
4	1.5 (0.06)	X	X[f]			UV

[a] WWTP = wastewater treatment plant, MGD = millions of gallons per day, UV = ultraviolet.
[b] Microfiltration followed by reverse osmosis.
[c] Activated sludge.
[d] Nutrient removal and effluent filtration.
[e] Alum flocculation.
[f] Trickling filter.

HPLC cleanup and ELISA analysis

Hydrolyzed and unhydrolyzed samples were subjected to high-pressure liquid chromatography (HPLC) to remove interfering compounds. A 250-mm Alltima C-18 column (5-μm particles, 9% carbon load, Alltech, Deerfield, IL, USA) was used with mobile phases consisting of 63% methanol/37% water or 43% acetonitrile/57% water at a flow rate of 1 ml/min. Concentrated standards (1.0 mg/L), which could be detected by UV absorbance at 220 and 280 nm, were injected before and after wastewater samples to confirm the retention time of hormones. The retention times for 17β-estradiol and 17α-ethinyl estradiol were approx. 17.6 and 17.2 min, respectively, with 63% methanol/37% water eluent and were approx. 16.5 and 21.6 min, respectively, with 43% acetonitrile/57% water eluent. The autosampler needle was washed with water twice after injection of the concentrated standards to prevent contamination of the samples. The ELISA analysis of deionized water samples, fractionated immediately after the concentrated standard was injected, indicated that carryover of the hormones did not occur.

For each concentrated extract, 60- to 90-μl aliquots were subjected to HPLC cleanup. One-minute eluent samples were collected and dried under vacuum or a gentle stream of nitrogen gas at room temperature. Dried HPLC fractions were resuspended in 200 μl of water and analyzed using enzyme-linked immunosorbent assay kits (Neogen, Lexington, KY, USA, for 17β-estradiol; Biopharm, Marshall, MI, USA, for 17α-ethinyl estradiol). Both ELISA kits are direct ELISAs containing polyclonal antibodies. The cross-reactivity of the antibodies to other hormones were reported by the manufacturers to be less than 1%. Overall, the concentration factors for ELISA analysis ranged from 225 to 1,500 for wastewater effluent samples and from 750 to 2,400 for surface water samples.

After adding the enzyme conjugates and chromophores supplied by the manufacturer, absorbance was measured at 630 and 450 nm using an automated microplate reader (Bio-Tek, Winooski, VT, USA). An incubation time of 60 min, which is longer than the 30 min recommended by the manufacturers, was employed for a few reasons: The extended time allowed sufficient color development and rendered the largest difference between a positive response and the background, the longer incubation time minimized assay-to-assay variability, and the entire analysis could be completed within a reasonable time (2.5–3.5 h). Calibration curves were obtained by analyzing standards of estrogenic hormones from 0.16 to 5 μg/L at six levels for each analysis. In the ELISA analysis, the absorbance signal is inversely related to the hormone concentration in a logarithmic manner. Calibration curves were obtained by plotting absorbance against log of hormone concentration using a second-order polynomial curve fitting program.

GC/MS/MS detection

To obtain a detectable signal during GC/MS/MS analysis, a higher preconcentration factor was needed. A total of 800 to 2,400 μl of concentrated sample extract were subjected to HPLC cleanup by repeated injections of 200-μl aliquots. The fractions containing hormones were combined and dried under a gentle stream of nitrogen gas. The HPLC fractions collected immediately before and after the hormone fraction also were collected and dried in a similar fashion. The dried HPLC fractions were resuspended in 200 μl of acetonitrile for derivatization. To prevent loss of hormones due to adsorption onto glass surfaces, all glassware was silanized with 5% chlorotrimethylsilane in toluene prior to derivatization. The mixture was derivatized with 50 μl of heptafluorobutyric anhydride (Aldrich, Milwaukee, WI, USA) at 50°C for 1.5 h. The derivatized samples were cooled to room temperature, dried under a gentle stream of nitrogen gas, and resuspended in 150 μl of isooctane (Aldrich) for GC/MS/MS analysis. Overall, the concentration factors for GC/MS/MS analysis ranged from 14,000 to 24,000 for wastewater effluents and from 32,000 to 64,000 for surface waters. A small amount of *p,p'*-DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) was added to samples after derivatization as an internal standard.

The derivatized 17β-estradiol was analyzed using a Finnigan GCQ ion trap GC/MS system (ThermoQuest, San Jose, CA, USA) with an AS2000 autosampler and a 30-m × 0.25-mm-ID × 0.25-μm-film-thickness Rtx-5 capillary column (Restek, Bellefonte, PA, USA). The GC conditions were as follows: injection port, splitless injection of 2 μl at 250°C; initial oven temperature, 75°C with a 1-min hold; programming rates, 20°C/min from 75 to 250°C, with a 5-min hold at 250°C; and 40°C/min from 250 to 290°C, with a 10-min hold at 290°C. The carrier gas (helium) was held constant at 1.2 ml/min. Ionization was achieved by electron ionization at 70 eV. The temperature of the ion source and the transfer line from GC to the mass spectrometer were 200 and 250°C, respectively. Under these conditions, the internal standard *p,p'*-DDE and 17β-estradiol derivative were eluted from the GC column at approx. 10.98 and 12.96 min, 250°C, respectively.

For quantification of 17β-estradiol, MS/MS analysis was conducted on the m/z 664 precursor ion with 0.7 eV collision energy to monitor the product ions of m/z 237, 409, and 450. The GC/MS/MS analysis was not conducted for 17α-ethinyl

Fig. 2. (**a**) Ultraviolet/visible absorption measured during high-performance liquid chromatography (HPLC) cleanup of samples collected from wastewater treatment plant (WWTP) 3 (March 31, 1998). (**b**) Enzyme-linked immunosorbent assay (ELISA) signal measured in HPLC fractions collected.

estradiol because the derivitization technique yielded poor recoveries for 17α-ethinyl estradiol. Calibration curves for quantification were produced by injecting standards of 17β-estradiol heptafluorobutyryl (HFB) derivative (3.8–106 µg/L at five levels) prepared along with each batch of samples.

RESULTS AND DISCUSSION

Sample cleanup, recoveries, and detection limits

Natural organic matter (NOM) can interfere with both ELISA and GC/MS analysis. Natural organic matter affects ELISA by adsorbing onto antibodies and other surfaces in the immunoassay system. In GC/MS analysis, NOM can produce interfering peaks, especially after derivatization. Therefore, it is necessary to remove NOM from the concentrated extracts prior to analysis. When extracts were fractionated by HPLC, interference corresponding to 3 to 10 ng/L of 17β-estradiol was detected by ELISA during the initial part of the chromatogram (Fig. 2). This signal was coincident with the elution of NOM, as indicated by UV absorption (Fig. 2). In samples from WWTPs 2 and 3, the ELISA interference and strong UV absorption were absent from HPLC fractions collected after approx. 10 min. In the sample from WWTP 1, an ELISA interference corresponding to approx. 6 ng/L of 17β-estradiol was detected at approx. 12 min. This interference was coincident with a relatively small peak of UV-absorbing material. With the exception of the fractions collected immediately before and after the hormones, the HPLC fractions from WWTP 4 and surface water samples were not analyzed for ELISA interference. The HPLC eluent for the entire chromatogram also was not analyzed by ELISA to assess interference during analysis of 17α-ethinyl estradiol.

A total of five HPLC fractions were analyzed for most samples (two before and two after the estrogenic hormone fraction as well as the hormone fraction). The ELISA analyses of the fractions before and after the hormone fraction typically exhibited a background signal corresponding to less than 20%

of the concentration in the hormone fraction. The background signal was consistent and reproducible. The sum of the apparent concentration detected in fractions collected before and after the hormone never exceeded 40% of the hormone concentrations detected in the hormone fraction. For standard analysis, we do not recommend the collection of more than five fractions per sample unless significant background signal is observed. Hormone concentrations were calculated by subtracting the average background signal and then adjusted according to the calculated recovery efficiencies. As stated previously, the signal to hormone concentration relationship in ELISA is a logarithmic function. Therefore, the method detection limits for both hormones, estimated as two times the background signal, were approx. 0.1 ng/L for wastewater effluents and 0.05 ng/L for surface waters.

To assess the potential for adsorption of dissolved hormones onto filters and sample containers, recovery experiments were performed by amending samples with hormones prior to filtration. The 17β-estradiol was added to unfiltered wastewater effluent from WWTP 1 at a concentration of 10 ng/L. After filtration, extraction, and cleanup, a recovery of 99% was obtained, indicating that sorption of hormones onto filters and sample containers was negligible. Furthermore, experiments performed to analyze losses during HPLC fractionation and blow-down steps indicate that losses during these steps are minimal.

Recovery experiments were conducted separately on estrogenic hormones and hormone conjugates. To determine hormone recovery, 3 to 10 ng/L of 17β-estradiol and/or 17α-ethinyl estradiol or 3 to 10 ng/L 17β-estradiol glucuronide and/or 17β-estradiol sulfate were added to filtered samples. Recoveries ranged from 44 to 117% for a total of 15 samples. The lowest recoveries were observed for the relatively polar hormone conjugate 17β-estradiol sulfate (mean recovery = 67 ± 11%), suggesting that some losses may occur as a result of incomplete extraction of the conjugate. The mean recovery of the other hormone conjugate, 17β-estradiol glucuronide, was 76 ± 25%. Recoveries were higher for 17β-estradiol (mean recovery = 79 ± 26%) and 17α-ethinyl estradiol (mean recovery = 75 ± 20%). Recoveries did not depend on the organic carbon content of the samples.

Estrogenic hormones in wastewater effluents

Results from ELISA analyses conducted at WWTPs 1 to 4 are summarized in Table 2 and Figure 3. In general, the concentrations of hormones were related to the sophistication of the treatment system. Concentrations of estrogenic hormones were highest in the effluent of WWTP 1, the plant that employs only primary and secondary treatment. The mean concentrations of 17β-estradiol and 17α-ethinyl estradiol were 3.8 and 2.0 ng/L, respectively. At WWTP 2, the plant equipped with biological nutrient removal and effluent filtration, the concentrations of 17β-estradiol and 17α-ethinyl estradiol were less than half of the concentrations measured at WWTP 1 (mean values of 0.8 and 0.3 ng/L, respectively). At WWTP 3, the advanced treatment plant, samples collected after microfiltration yielded concentrations of 17β-estradiol and 17α-ethinyl estradiol similar to those detected in the effluent of WWTP 2 (mean values of 1.4 and 0.14 ng/L, respectively). The lowest concentrations of hormones were observed in the reverse osmosis effluent from WWTP 3 with hormone concentrations near or below the detection limits (0.1 ng/L). Results of the sample from WWTP 4, a system that employs a trickling filter,

Table 2. Results of enzyme-linked immunosorbent assay (ELISA)[a]

Sample	Effluent	Date	E_2 (ng/L)[b]	Recovery (%)	EE_2 (ng/L)[c]	Recovery (%)
WWTP 1	Secondary	12/9/97	3.91 ± 0.29	72 ± 5	NM	NM
		1/27/98	2.75	78	NM	NM
		3/11/98	4.05 ± 0.22	81	1.54 ± 0.24	74
		6/10/99	3.68 ± 1.27	68	2.42 ± 0.60	NM
		Average	3.76 ± 0.67	75 ± 6	1.98 ± 0.64	NA
WWTP 4	Secondary	9/20/99	0.20 ± 0.03	102	0.66 ± 0.19	82
WWTP 2	Tertiary	6/19/98	0.70 ± 0.32	49	0.19 ± 0.10	71
		7/23/98	0.38 ± 0.15	117	0.26 ± 0.15	110
		9/28/98	1.01 ± 0.46	62	0.49 ± 0.21	75
		Average	0.77 ± 0.43	76 ± 36	0.33 ± 0.20	85 ± 22
WWTP 3	Microfiltration	3/31/98	2.65	51	0.16	76
		12/14/99	0.72 ± 0.15	92	0.13 ± 0.03	NM
		Average	1.36 ± 1.12	72 ± 29	0.14 ± 0.03	NA
WWTP 3	Reverse osmosis	3/31/98	<0.1	63	<0.1	51
		12/14/99	0.32 ± 0.01	63	<0.1	82
		Average	0.24 ± 0.15	63 ± 1	<0.1	67 ± 22
Engineered wetland[d]		9/20/99	0.05 ± 0.03	108	0.07 ± 0.01	89
Colorado River, near San Diego		12/15/99	0.80 ± 0.24	101	<0.05	NM
Sacramento River Delta		8/26/99	0.08 ± 0.02	91	<0.05	54

[a] WWTP = wastewater treatment plant, NM = not measured, NA = not applicable.
[b] E_2: 17β-estradiol.
[c] EE_2: 17α-ethinyl estradiol.
[d] Receives the secondary effluent from treatment plant 4.

yielded lower concentrations of hormones (0.2 and 0.7 ng/L for 17β-estradiol and 17α-ethinyl estradiol, respectively) than WWTP 1.

For each WWTP, the concentrations of estrogenic hormones were consistent in samples collected on different days. With the exception of several samples from the reverse osmosis system, 17β-estradiol was present at concentrations at least two times higher than the method detection limit (0.1 ng/L).

The GC/MS/MS confirmatory analyses were conducted on effluent samples collected from WWTP 1 (June 10, 1999) and WWTP 4 (September 20, 1999). The full-scan chromatogram of 17β-estradiol standard yielded only one significant peak. The electron ionization fragmentation of this peak yielded ions at m/z 237 (100%), 409 (33%), 450 (36%), and 664 (13%). These results confirm that the derivatization conditions em-

ployed produce only the 17β-estradiol-3,17-$(HFB)_2$ derivative. Tandem mass spectrometry analysis conducted on the molecular ion of 17β-estradiol-3,17-$(HFB)_2$ (m/z = 664) yielded product ions of m/z 237, 409, and 450. The signal-to-noise ratio for 17β-estradiol in all samples analyzed was greater than 12. Analyses by GC/MS/MS yielded concentrations of 17β-estradiol comparable to those determined by ELISA. Analysis of a sample collected from WWTP 1 on June 10, 1999, yielded 3.68 ± 1.27 ng/L of 17β-estradiol when analyzed by ELISA versus 3.9 ± 1.4 ng/L when analyzed by GC/MS/MS. Analysis of a sample collected from WWTP 4 on September 20, 1999, yielded 0.20 ± 0.03 ng/L of 17β-estradiol when analyzed by ELISA versus 0.27 ± 0.20 ng/L when analyzed by GC/MS/MS. The detection limits of GC/MS/MS, estimated as three times the signal-to-noise ratio of the baseline, are approx. 0.2 to 0.4 ng/L, depending on the sample matrices and the performance of GC/MS/MS. Furthermore, 17β-estradiol was not detected in the HPLC fractions collected before or after the 17β-estradiol fraction.

Enzymatic hydrolysis was performed on 70% of the wastewater effluent samples. Hormone conjugates accounted for less than 2% of the hormones in all samples analyzed. The low concentrations of conjugated hormones are consistent with enzymatic hydrolysis of conjugates in the wastewater collection and treatment system. Previous studies indicate that glucuronidase and sulfatase enzymes in the large intestine rapidly hydrolyze conjugated hormones [9]. Hydrolysis of conjugated pharmaceuticals also has been observed in manure [19]. Although estrogenic hormones are excreted mainly in conjugated forms, they are converted into active hormones prior to effluent discharge.

Most of our measurements of estrogenic hormones in conventional wastewater treatment plants are comparable to results from other studies (Table 3). Excluding the advanced wastewater treatment plants, our median concentration of 17β-estradiol is comparable to the median concentrations determined by GC/MS/MS and GC/MS. Snyder et al. [17] reported a median concentration of 0.7 ng/L for 17β-estradiol in waste-
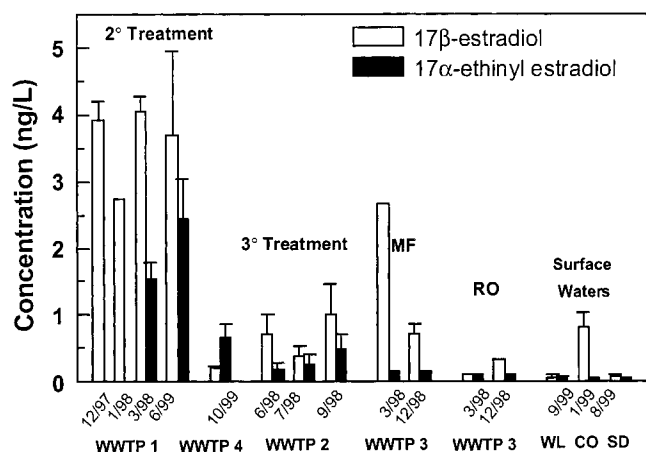

Fig. 3. Concentrations of estrogenic hormones measured by enzyme-linked immunosorbent assay (ELISA). Error bars indicate the standard deviation among replicate samples. MF = microfiltration, RO = reverse osmosis, WL = an engineered wetland that receives the secondary effluent from wastewater treatment plant (WWTP) 4, CO = Colorado River near San Diego, SD = Sacramento River Delta near Patterson Pass. 17α-ethinyl estradiol was not measured at December 1997 and January 1998.

Table 3. Ranges and median concentrations of estrogenic hormones in wastewater effluents (data from the advanced wastewater treatment plant are excluded)

| | Concentration (ng/L) | | | | | | | | | |
| | Desbrow et al. [5] | | Ternes et al. [14] | | Belfroid et al. [15] | | Snyder et al. [17] | | This study | |
Compound	Range	Median	Range	Median[a]	Range	Median	Range	Median	Range	Median
17β-estradiol	2.7–12	5.9	<1–64	6, <1	<0.4–12	0.7	<0.1–3.7	0.7	0.2–4.1	1.9
17α-ethinyl estradiol	<0.2–4.3	<0.2	<1–42	9, 1	0.2–7.5	<0.3	<0.05–0.8	0.15	0.2–2.4	0.6

[a] Median values calculated separately for data from German and Canadian wastewater treatment plants.

water effluent using radioimmunoassay. The highest concentrations detected by ELISA (4.1 ng/L in this study) and radioimmunoassay (3.7 ng/L [17]) are considerably lower than the highest concentrations determined by GC/MS or GC/MS/MS. Since some results from GC/MS analyses are considerably higher than estimated influent concentrations [13], it is possible that GC/MS sometimes overestimates hormone concentrations in wastewater. Furthermore, none of the wastewater samples, excluding reverse osmosis effluent, analyzed in this study contained hormones at concentrations below the method detection limits (0.1 ng/L). In contrast, more than 50% of samples analyzed in previous studies using GC/MS or GC/MS/MS were below detection limits.

On the basis of hydrophobicity (the octanol/water partition coefficient for 17β-estradiol is approx. 15,000 [20]), estrogenic hormones should have some affinity for particles in wastewater. For example, using the octanol/water partition coefficient to estimate the linear distribution coefficient, K_D [20], we estimate that approx. 70% of the 17β-estradiol will be associated with particles during activated sludge treatment (assuming 2 g/L volatile suspended solids). The observation that 17β-estradiol concentrations measured with ELISA were never more than approx. 25% of the expected influent concentrations supports the possibility that the relatively high concentrations of hormones reported in some studies could be an artifact.

The incomplete removal of estrogenic hormones during secondary wastewater treatment is not surprising, given data on the removal of similar compounds during secondary wastewater treatment. For example, Elhmmali et al. [21] reported the removal of approx. 50% of the dissolved deoxycholic acid, a bile acid with a structure similar to steroid hormones, during secondary treatment. Although the biological transformation of hormones has been reported [10,22,23], these laboratory experiments were performed at hormones concentrations many orders of magnitude greater than those present in wastewater. Biotransformation may not be as important in wastewater treatment plants because transformation rates often decrease at lower concentrations. Therefore, the main removal mechanism for hormones may be adsorption onto particles.

Estrogenic hormones in surface waters

Estrogenic hormones were detected in all the surface waters samples at concentrations below 1 ng/L (Table 2 and Fig. 3). Analysis of a sample collected from an engineered wetland that receives the secondary effluent from WWTP 4 indicated that the concentration of 17β-estradiol decreased from 0.20 ± 0.03 ng/L in the wastewater effluent to 0.05 ± 0.03 ng/L in the outlet of the wetland. The concentration of 17α-ethinyl estradiol in the wetland decreased from 0.66 ± 0.19 ng/L to 0.07 ± 0.01 ng/L. The estrogenic hormones may be removed by a combination of physical and biological processes during its passage through the wetland. However, additional measurements are needed to assess temporal variation in hormones discharged by the wastewater treatment plant.

Water from the Sacramento Delta contained approx. 0.08 ± 0.02 ng/L of 17β-estradiol, while water collected from the Colorado River contained a higher 17β-estradiol concentration (0.80 ± 0.24 ng/L). Concentrations of 17α-ethinyl estradiol in both waters were below the detection limits (0.05 ng/L). Compared to wastewater effluent, surface water generally has less natural organic matter, and a larger volume can be extracted, resulting in a lower detection limit.

Enzymatic hydrolysis for hormone conjugates was con-

Table 4. Ranges and median concentrations of estrogenic hormones in surface waters

	Concentration (ng/L)							
	Ternes et al. [14]		Belfroid et al. [15]		Snyder et al. [17]		This study	
Compound	Range	Median	Range	Median	Range	Median	Range	Median
17β-estradiol	<0.5	<0.5	<0.3–5.5	<0.3	<0.1–2.7	0.7	0.05–0.8	0.08
17α-ethinyl estradiol	<0.5	<0.5	<0.1–4.3	<0.1	<0.05–0.5	<0.05	<0.05–0.07	<0.05

ducted on the Colorado River samples. Similar to the results of wastewater, no significant concentrations of hormone conjugates were detected ($<1\%$ of the hormones). These results are consistent with hydrolysis of the hormone conjugates prior to wastewater discharge.

Confirmatory analysis by GC/MS/MS was conducted on the Sacramento Delta sample. Measured concentrations of 17β-estradiol were considerably higher than those determined by ELISA (0.38 ± 0.21 ng/L from GC/MS/MS vs 0.08 ± 0.02 ng/L for ELISA). This discrepancy could be related to problems with GC/MS/MS quantification at concentrations close to the method detection limit. No 17β-estradiol was detected in the HPLC fractions collected before and after the 17β-estradiol fraction. In previous studies [14,15], hormone concentrations in most surface water samples ($>70\%$) were below the detection limits of GC/MS/MS method (Table 4).

CONCLUSIONS

Results of this study indicate that the concentrations of estrogenic hormones 17β-estradiol and 17α-ethinyl estradiol discharged by conventional wastewater treatment plants range from approx. 0.2 to 4.1 ng/L. For comparison, vitellogenesis in fish has been observed at hormone concentrations as low as 1 ng/L [1,6]. Concentrations of estrogenic hormones in reverse osmosis effluent from an advanced WWTP were <0.4 ng/L. Compared to GC/MS/MS, the ELISA technique has lower detection limits, requires smaller concentration factors, and is less susceptible to matrix interference. The ELISA technique, coupled with confirmatory analysis with GC/MS/MS, provides a sensitive means of quantifying hormones in wastewater and surface waters. Further research is needed to assess engineering controls that can be used to remove hormones and to assess the fate of hormones after they are discharged.

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