The Fate of Estrogenic Hormones in an Engineered Treatment Wetland with Dense Macrophytes

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ABSTRACT: Recently, the estrogenic hormones 17β-estradiol (E_2) and 17α-ethinyl estradiol (EE_2) have been detected in municipal wastewater effluent and surface waters at concentrations sufficient to cause feminization of male fish. To evaluate the fate of steroid hormones in an engineered treatment wetland, lithium chloride, E_2, and EE_2 were added to a treatment wetland test cell. Comparison of hormone and tracer data indicated that 36% of the E_2 and 41% of the EE_2 were removed during the cell's 84-h hydraulic retention time (HRT). The observed attenuation was most likely the result of sorption to hydrophobic surfaces in the wetland coupled with biotransformation. Sorption was indicated by the retardation of the hormones relative to the conservative tracer. Biotransformation was indicated by elevated concentrations of the E_2 metabolite, estrone. It may be possible to improve the removal efficiency by increasing the HRT or the density of plant materials. *Water Environ. Res.,* **77,** 24 (2005).

KEYWORDS: estradiol, ethinyl estradiol, engineered treatment wetlands, endocrine disruption.

Introduction

The estrogenic hormones 17β-estradiol (E_2) and 17α-ethinyl estradiol (EE_2) are discharged to the environment mainly through municipal wastewater effluent and agricultural runoff (Belfroid et al., 1999; Desbrow et al., 1998; Huang and Sedlak, 2001; Lange et al., 2002; Ternes et al., 1999b). These hormones are potent endocrine disruptors capable of causing feminization of male fish at concentrations as low as 1 ng/L (Lange et al., 2002; Routledge et al., 1998; Thorpe et al., 2001). Concentrations of hormones above 1 ng/L have been observed both in municipal wastewater effluents and effluent-dominated receiving waters (Desbrow et al., 1998; Ferguson et al., 2001; Kolodziej and Sedlak, 2004; Kolodziej et al., 2003; Purdom et al., 1994; Snyder et al., 1999; Spengler et al., 2001; Ternes et al., 1999b). Under certain conditions, advanced wastewater treatment processes (e.g., reverse osmosis and treatment with granular activated carbon) can reduce hormone concentrations to levels that no longer induce feminization of fish (de Rudder et al., 2004; Huang and Sedlak 2001; Schafer et al., 2003). However, these technologies are expensive and generally are not applied to the treatment of wastewater that is not intended for potable reuse.

To minimize the potential risks to aquatic ecosystems from the discharge of municipal wastewater effluent and agricultural runoff, inexpensive treatment processes capable of removing trace concentrations of hormones are needed. Engineered treatment wetlands are already in widespread use for the treatment of municipal wastewater effluent and agricultural runoff (Bachand and Horne 2000a; Kadlec and Hey, 1994; Mitsch et al., 2000). Treatment wetlands effectively remove a variety of contaminants, including nutrients, pesticides, and pollutant metals (Alvord and Kadlec, 1995; Bachand and Horne, 2000b; Schulz and Peall, 2001; Schulz et al., 2001). Among the previously studied contaminants, moderately hydrophobic pesticides exhibit the most similar chemical properties to estrogenic hormones and, therefore, could provide insight to the potential fate of hormones in engineered treatment wetlands. Several of these pesticides undergo attenuation in engineered wetlands by processes including sorption to surfaces, biodegradation, and photolysis (Alvord and Kadlec, 1996; Miller and Chin, 2002; Moore et al., 2000; Runes et al., 2001). Therefore, similar mechanisms could result in hormone attenuation in engineered wetlands.

Most surface-flow engineered treatment wetlands have a water depth of less than one meter flowing through a bed of emergent macrophytes and organic sediments. The resulting high density of organic surfaces makes it likely that nonpolar compounds will undergo sorption in wetlands. For example, when chlorpyrifos (log K_{ow} = 5.8) was added to the inlet of an engineered wetland, it was retained on surfaces in the first 30 meters downstream in the wetland (Moore et al., 2002). Studies conducted using sediments from rivers indicate that estrogenic hormones (log K_{ow} = 4–4.2) can sorb to suspended and bed sediments (Bowman et al., 2002; Holthaus et al., 2002; Lai et al., 2000; Yu et al., 2004). Therefore, in engineered wetlands, sorption is a potentially important mechanism for removal of hormones from the aqueous phase.

Although sorption may result in the removal of hormones from the aqueous phase, the compounds will eventually pass through the wetland if transformation does not occur. Engineered treatment wetlands provide an environment with high surface areas of biofilms that are conducive to biotransformation of organic compounds. Sorption coupled with biodegradation has been invoked as an explanation for the removal of the pesticides atrazine, azinphos-methyl, and chlorpyrifos in wetlands (Alvord and Kadlec, 1995 and 1996; Moore et al., 2002; Schulz and Peall, 2001; Schulz et al., 2001). Furthermore, experiments using columns packed with freshwater sediments demonstrate sorption coupled with degradation of E_2 at rates significantly faster than in columns packed with sand or soil (up to 0.075 h^{-1}) (Das et al., 2004). Bacteria present in municipal wastewater treatment systems (Layton et al., 2000; Ternes et al., 1999a) and in surface waters (Jurgens et al., 2002) also can transform E_2 to estrone (E_1) and other metabolites. Furthermore, in certain circumstances, wetland treatment has been shown to mitigate *in vitro* estrogenic activity. However, in the same study, *in vivo* estrogenic activity was not significantly decreased (Xie et al., 2004). The synthetic hormone EE_2, which is more resistant to biodegradation than E_2 (Ternes et al., 1999b) can be transformed by nitrifying bacteria (Vader et al., 2000).

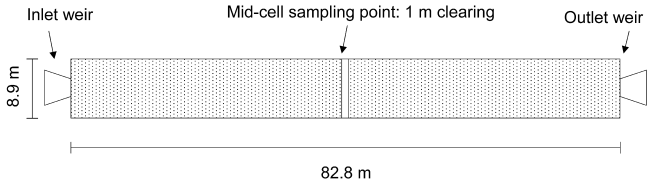

Figure 1—Schematic representation of the wetland cell used in this study. The shaded area represents locations with dense growth of cattails (*Typha spp.*) and bulrushes (*Scirpus spp.*).

To assess the importance of sorption and biotransformation to the attenuation of hormones in engineered treatment wetlands, a study was conducted in a 750-m^2 treatment cell at the Orange County Water District's (OCWD's) Prado Engineered Treatment Wetland (Riverside County, California). To facilitate the detection of hormone attenuation, flow to one of six parallel wetland test cells was amended with of lithium chloride (LiCl), E_2, and EE_2 at concentrations significantly above their typical background levels. By observing retardation of the hormones relative to the conservative tracer (i.e., lithium), it was possible to distinguish sorption from biotransformation and to estimate the extent of hormone removal in the wetland. Additional evidence for hormone biotransformation was provided by simultaneous measurements of E_1.

Methodology

Materials. All chemicals were obtained from Fisher Scientific (Pittsburgh, Pennsylvania) at the highest possible purity, unless otherwise specified. Hormones (E_2 and EE_2, purity 98% minimum) were obtained from Sigma (St. Louis, Missouri). Deionized water required for stock solutions and quality control samples was produced with a Nanopure system (Barnsted, Dubuque, Iowa), stored in glass bottles, and transported to the field site. Enzyme-linked immunosorbent assay (ELISA) kits for determination of E_2 and EE_2 were obtained from r-Biopharm (Marshall, Michigan).

Experimental Conditions. The field study was conducted in the test cells adjacent to OCWD's Prado Engineered Treatment Wetland, which is located in Riverside County, California (33° 53′ N, 117° 58′ W). The water enters the wetland from the Santa Ana River, which has a dry weather base flow of approximately 3.6 m^3/s (80 million gal/d) at the entrance to the wetland. The dry weather base flow is derived principally from tertiary wastewater effluent discharged upstream by the cities of Riverside and San Bernadino. Approximately half of the base-flow of the Santa Ana River is diverted into the Prado Wetland as part of a program designed to remove nitrogen by denitrification (Bachand and Horne, 2000a). Approximately 0.005 m^3/s of the flow in the wetland is diverted to six parallel wetland test cells.

On the basis of results from a preliminary tracer test at the wetland (Gray, 2003), the second cell from the north, which is referred to as cell M2, was chosen for the study (Figure 1). The wetland cell was 82.8 × 8.9 m (Bachand and Horne, 2000a), and the water level was maintained at a depth of 0.5 m throughout the study, for a total volume of 368 m^3. The cell contained dense growth of emergent vegetation, primarily cattails (*Typha latifolia* and *Typha domingensis*) and bulrushes (*Scirpus acutus*, *Scirpus validus*, and *Scirpus californicus*). To facilitate sampling and promote mixing, a 0.7-m wide swath of vegetation was cleared from the cell before the study. This area, which is referred to as the mid-cell sampling location, was located midway between the inlet and exit weirs.

On October 14, 2002, 500 g LiCl were dissolved in 12 L of water collected from the inlet of cell M2. After the LiCl was dissolved, 100 mL of a solution containing 290 mg/L EE_2 and 270 mg/L E_2(in methanol) were added to the LiCl solution. This resulted in a solution containing 7.0 g/L methanol, 4.20 g/L Li^+, 2.40 mg/L EE_2, and 2.25 mg/L E_2, which was added directly to the inlet weir of cell M2 over a period of three minutes. After dilution in the inlet weir, the concentration of methanol was low enough that it should not have affected biological oxygen demand in the wetland significantly (i.e., ignoring biotransformation, the methanol concentration would have been less than 0.5 mg/L at the midpoint).

After addition of the conservative tracer and hormones, grab samples were collected from the wetland periodically between October 14 and 25. Samples were collected from the midpoint and the outlet weir of cell M2 to assess hormone attenuation. Samples also were collected from the inlet to cell M2 and from the outlet from a test cell that did not receive any treatment (cell M5) to assess background levels of hormones and Li^+. Grab samples were collected directly from the inlet and outlet weirs, where vigorous mixing assured representative samples. At the midpoint, a 1.5-m pole with a 1-L glass beaker attached to the end was used to collect samples from a depth of approximately 0.2 m as far as possible from the bank of the wetland.

Samples were collected at approximately 4-h intervals for the first 3 days of the study and 2 to 3 times daily thereafter. Three separate sample aliquots were collected at each sampling point. The first was a 3-L sample collected in a 12-L fluorinated high-density polyethylene (HDPE) jerrican for hormone analysis and determination of total suspended solids (TSS). The second sample was a 100-mL sample collected in a 125-mL HDPE container for Li^+ and nitrate analysis. The third sample was a 30-mL aliquot collected in a glass screw top jar for dissolved organic carbon (DOC) analysis. The hormone and TSS samples were filtered and extracted immediately at OCWD's on-site laboratory. The Li^+ and nitrate samples were refrigerated at 4° C within 15 minutes of collection, and the DOC sample was frozen within 15 minutes of collection.

Chemical Analyses. Within 30 minutes of sample collection, 2 L of the hormone sample was filtered through preweighed 90 mm, 0.5-μm glass fiber filters (Millipore, Bedford, Massachusetts) for TSS analysis. Hormone extraction was performed by decanting 250 mL of the filtrate into a 500-mL Erlenmeyer flask. The subsample was amended with 250 μL of 100 μg/L mesterolone (i.e., the surrogate standard for gas chromatography/tandem mass spectrometry [GC/MS/MS] analysis of hormones). This solution then was passed through a 47-mm Empore C_{18} solid-phase extraction (SPE) disk (Millipore) under vacuum. Before extraction, the SPE disks were preconditioned with 10 mL of methanol followed by 10 mL of deionized water. After extraction, the hormones were eluted from the disks with 15 mL of 95% methanol in water. This extract was stored in 30-mL glass screw-cap vials at −5° C until analysis.

Quality assurance and quality control samples were analyzed throughout the study. For hormone recovery analyses, 30-mL aliquots of wetland water from the outlet of the adjacent wetland cell (cell M3) were amended with E_2 and EE_2 to achieve a concentration of 120 ng/L. These samples were filtered and extracted in the manner described above. Eight spike recovery samples were analyzed during the study, and the average recovery of E_2 and EE_2 was 80.2 ± 10.3%. Six of the samples were analyzed in duplicate, and their deviations never exceeded 30%. Four, 250-mL deionized water blanks were filtered, extracted, and analyzed. Hormones were never detected in the blanks.

Within two weeks of collection, the hormone extracts were dried in a vacuum oven at 50° C and resuspended in 1-mL of deionized water. To separate E_2 and EE_2 from organic matter capable of interfering with ELISA analysis, a set of 20 extracts were subjected to gel-permeation chromatography (GPC) and reversed-phase high-performance liquid chromatography (HPLC). A 250 × 4.6-mm ID Alltech (Pittsburgh, Pennsylvania) Macrosphere 300 Å column was used for GPC separations. The mobile phase consisted of 15% acetonitrile in pH 3 phosphate buffer (5 mM) at a flowrate of 0.5 mL/min. After GPC separation, the hormone-containing fraction was collected and retained for further purification (by reversed-phase HPLC) and analysis. For reversed-phase cleanup, a 250 × 4.6 mm ID Alltech Alltima C_{18}-LL end-capped column with a mobile phase of 42% acetonitrile in water was used, as described elsewhere (Huang and Sedlak, 2001). After the first set of extracts were analyzed, it was determined that the concentration of interfering compounds present in the extracts was low relative to hormone concentrations, and the remainder of the extracts were subjected only to reversed-phase cleanup. After all cleanup steps were complete, sample concentration factors ranged from approximately 50 to 200, corresponding to a limit of quantification (LOQ) for the ELISA method of 2 ng/L.

Hormone concentrations also were measured in samples collected from the Prado wetland, the Santa Ana River (upstream and downstream of the wetland), and several wastewater treatment plants (WWTPs), which discharge effluent to the Santa Ana River or one of its tributaries between 1999 and 2000. For these samples, 4-L aliquots of water were collected in fluorinated HDPE containers and filtered through 0.5-μm glass-fiber filters before extraction using 90-mm Empore SPE disks. After the disks were rinsed with a 30% methanol solution and eluted with a 95% methanol solution, the resulting extracts were purified using GPC and reversed-phase HPLC and analyzed by ELISA in the same manner described above.

For confirmation of E_2 concentrations and detection of E_1, 0.4 to 0.5-mL sub-samples from 19 of the SPE extracts were subjected to GC/MS/MS analysis as described elsewhere (Kolodziej et al., 2003). The recovery of mesterolone, the surrogate standard, was lower than expected (approximately 10%), presumably because of an error in the sample preparation procedure that resulted in the presence of trace amounts of water in the extracts that were subjected to derivitization. Because the effect of trace water on the efficiency of the derivitization of the surrogate and E_2 and E_1 were probably different, the reported values have considerable uncertainty and should be used only as qualitative indicators of hormone presence.

Other water quality parameters were analyzed using established protocols. Lithium samples were acidified to 0.1 N with concentrated nitric acid, diluted 4 to 32 times, and analyzed by graphite-furnace atomic absorption spectroscopy (APHA et al., 1999). The LOQ for Li^+ was approximately 2.5 μg/L. Two-liter aliquots of wetland water were filtered for TSS analysis (APHA et al., 1999). Nitrate was quantified according to APHA et al. (1999) using a Dionex DX-120 ion chromatograph (Dionex, Sunnyvale, California), and DOC was quantified according to the manufacturer's instructions using a Shimadzu TOC-5000A total organic carbon analyzer (Shimadzu, Columbia, Maryland).

Results

Conservative Tracer Analysis. During the study period, concentration profiles of the conservative tracer, lithium (Figure 2), were used to construct a residence time distribution that was consistent with the hydraulic model of plug-flow with dispersion. The wetland hydraulic retention time (HRT) and its variance (σ^2) were calculated from the residence-time distribution as described by Fogler (1998), after subtracting the 5 μg/L background concentration of Li^+. At the midpoint of the cell, the estimated HRT was 37 h and, at the end weir, HRT was 84 h. The values of σ^2 were 380 and 1200 h^2, respectively.

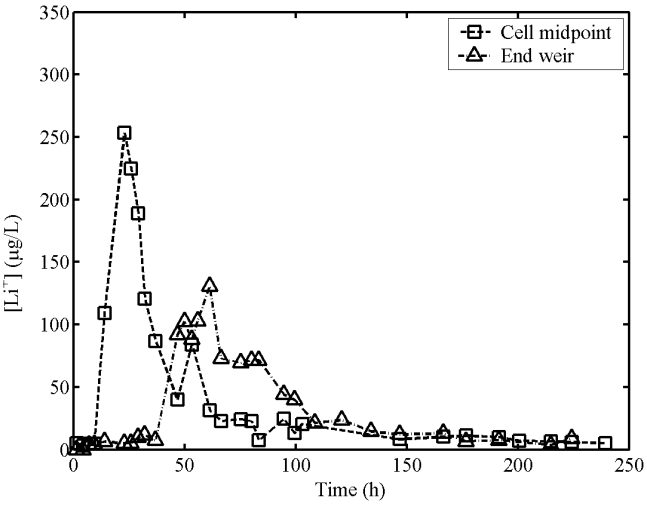

Figure 2—Concentration profile of lithium measured in the experimental wetland cell after the addition of hormones and lithium to the inlet weir. Squares indicate observations at cell's midpoint, while triangles represent observations at the end weir.

The average flowrate through the wetland (Q) then was calculated from the residence time distribution using the following expression:

$$Q = \frac{M}{\int_0^\infty C(t)dt} \tag{1}$$

Where M equals the total mass of lithium added to the cell (i.e., 50 g) and the integral term was approximated from the lithium concentration profile using the trapezoidal rule.

The average flowrate through the wetland was 1.5×10^{-3} m^3/s (5400 L/h). The flowrate calculated from the residence-time distribution was consistent with estimates of the flow coming into the wetland cell at the inlet weir.

The Peclet number (*Pe*) compares the importance of advection to dispersion in the wetland. It can be calculated as follows:

$$Pe = \frac{u \cdot L}{D} \tag{2}$$

Where

u = average velocity in the wetland,
L = length of the wetland cell, and
D = dispersion coefficient.

To determine the value of D analytically, the residence time distribution in the wetland must be normally distributed. Because this was not the case, it was necessary to estimate *Pe* empirically from the estimated mean HRT and σ^2 (Fogler, 1998).

$$\frac{\sigma^2}{HRT^2} = \frac{2}{Pe} + \frac{8}{Pe^2} \tag{3}$$

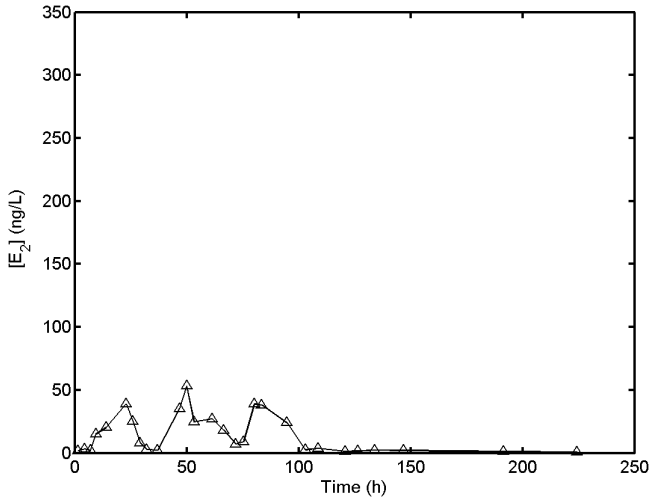

Figure 3—Concentration profile of 17β-estradiol (E_2) at the midpoint of the wetland cell.

During the course of the study, *Pe* was 9 at the wetland mid-point and 13 at the end weir. These values correspond to average dispersion coefficients of 1.2×10^{-3} m^2/s, calculated at the midpoint and end weir, and indicate a significant departure from plug-flow conditions.

Hormone Analysis. Concentrations of EE_2 were significantly higher than E_2 at the midpoint (Figures 3 and 4). The maximum observed concentrations at the midpoint were 53.1 ng/L E_2 at 50 h after hormone addition and 320 ng/L of EE_2 at 61 h after hormone addition. The first detection of hormones at concentrations above background occurred approximately 10 h after the addition of hormones to the wetland, while elevated concentrations of Li^+ were not observed until after 23 h. Three distinct EE_2 peaks were observed at 10, 26, and 61 h after hormone addition. The E_2 concentration profile was similar to that of EE_2, with three peaks of E_2 detected at 23, 50, and 83 h after hormone addition.

Concentrations of E_2 and EE_2 at the end weir were significantly lower than those encountered at the midpoint (Figures 5 and 6). The maximum EE_2 concentration of 116 ng/L was observed after 53 h, while the maximum E_2 concentration of 39 ng/L was observed after 47 h. Pulses of EE_2 were observed at 9.5, 53, and 95 h after hormone addition, while E_2 maxima were observed between 30 and 150 h. For comparison, the peak concentration of Li^+ occurred after 61 h.

Hormone concentrations in the wetland inlet and in the untreated wetland cell were considerably lower than those observed in the test cells. Hormones were never detected above the limit of quantification (2 ng/L) in samples collected from the untreated wetland cell (n = 10) or from the wetland influent (n = 10) throughout the duration of the study. During a survey of the Prado Wetland conducted in July 2000, the median concentration of E_2 was 0.20 ng/L, and the maximum concentration was 7.6 ng/L. In September and November 1999, E_2 was measured in the Santa Ana River and in several WWTPs that discharge effluent to the river. Concentrations were almost always below 2 ng/L (Table 1). During the same surveys,

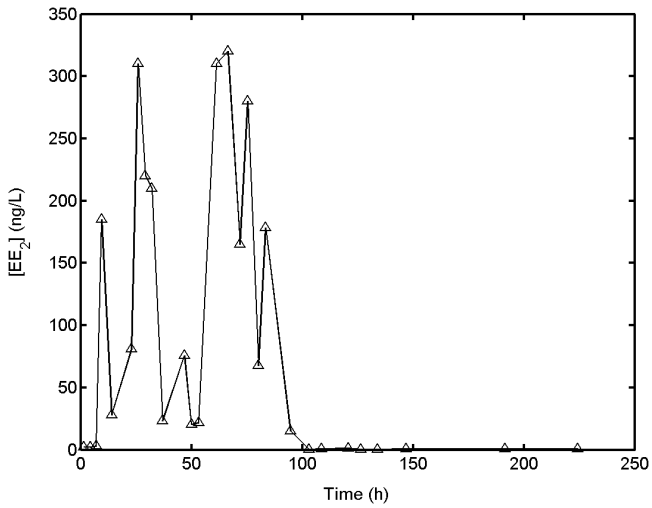

Figure 4—Concentration profile of 17α-ethinyl estradiol (EE_2) at the midpoint of the wetland cell.

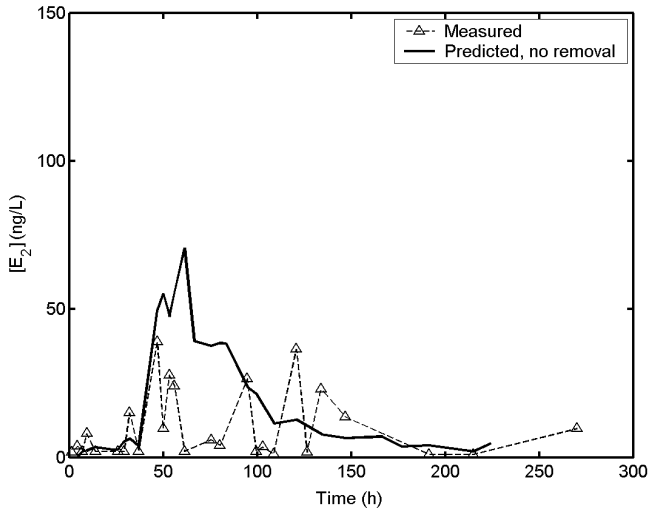

Figure 5—Concentration profile of 17β-estradiol (E_2) at the end weir. The solid line represents the expected E_2 concentration in the absence of retardation or attenuation.

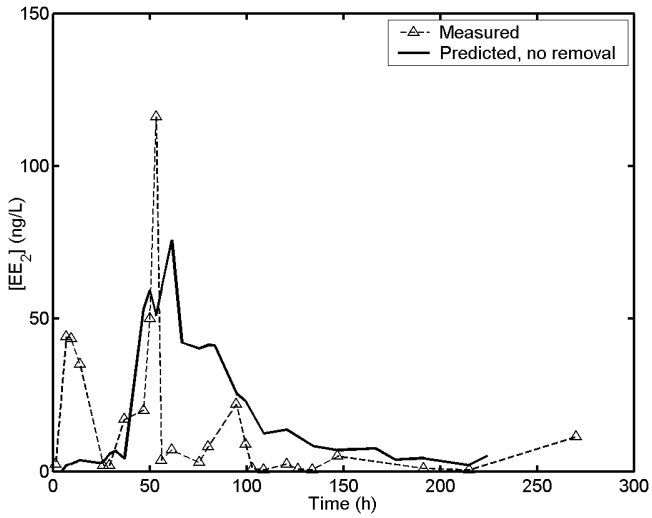

Figure 6—Concentration profile of 17α-ethinyl estradiol (EE_2) at the end weir. The solid line represents the expected EE_2 concentration in the absence of retardation or attenuation.

Table 1—17β-estradiol (E_2) concentrations (ng/L) measured in Prado Wetland and its source waters.

Site	Date	n	LOQ[a]	E_2 percentile 10th	Median	90th
Santa Ana R[b]	September 13, 1999	7	0.2	0.2	0.8	1.6
Santa Ana R	November 16 to 18, 1999	10	0.1	<0.1	0.7	1.3
SA WWTPs[c]	November 16 to 18, 1999	3	0.1	0.2	0.2	0.5
Prado Wetland	July 26, 2000	7	0.05	0.05	0.2	7.6

[a] Limit of quantification.
[b] Santa Ana River.
[c] Wastewater treatment plants discharging to Santa Ana River.

Table 2—Water quality parameters measured in Prado Test Cell M2.

Time (h)	pH	$[NO_3]$[a]	TSS[b]	[DOC][c]
Mid-cell				
14	7.3	13.2	<5	2.4
23	7.1	12.1	5	2.5
50	7.4	10.9	10	3.7
53	7.3	7.2	5	5.2
End weir				
4.5	7.0	4.2	5	7.0
7	7.5	3.4	<5	3.4
26	7.3	6.2	5	3.7
29	6.9	5.6	5	3.4
53	7.1	4.8	<5	3.5

[a] Nitrate concentration (mg/L).
[b] Total suspended solids (mg/L).
[c] Dissolved organic carbon (mg/L).

E_1 concentrations, quantified by GC/MS/MS, typically ranged from 5 to 10 ng/L in the wetland and in the Santa Ana River (n = 10, LOQ = 1 ng/L). The samples collected during the previous surveys were not affected by the presence of water during derivitization and therefore are quantitatively accurate.

Confirmatory analyses of E_2 and analysis of E_1 by GC/MS/MS were performed at the midpoint and at the end weir. These analyses indicated the presence of E_1 and E_2 in five of the eighteen samples analyzed. Concentrations ranged from 12 to 35 ng/L, and hormones were not detected by GC/MS/MS in samples collected before or after the pulse detected by the ELISA technique. However, as mentioned previously, the recovery of mesterolone (the surrogate standard) was low, and the results of the confirmatory analyses are useful mainly for qualitative purposes.

The concentrations of certain ancillary water quality parameters, including nitrate, TSS, and DOC, in selected samples from the test cell are summarized in Table 2.

Discussion

As with any field study in which complex analytical techniques are used to study biological and chemical processes, the data from the hormone addition study are complicated and must be interpreted by comparing the data with expectations based on theory. In the following sections, the data are evaluated to assess the relative importance of sorption and biotransformation in the wetland.

Hormone Mass Recovery. Assuming that lithium behaved conservatively, the fraction of hormones recovered at the midpoint and end weir can be calculated by comparing the lithium concentration profile to the hormone profiles. At the start of the experiment, 50 g of lithium, 27 mg of E_2, and 29 mg of EE_2 were added to the inlet weir. In the absence of attenuation, the ratio of the total E_2 recovered to total lithium recovered would be 5.4×10^{-4} (i.e., 27 mg per 50 g). Likewise, the ratio of the total EE_2 recovered to total lithium recovered would be 5.8×10^{-4}.

The mass of Li^+, E_2, and EE_2 recovered in the wetland cells was calculated by rearranging eq 1, as follows:

$$M = Q \cdot \int_0^\infty C(t)dt \tag{4}$$

The integral term in eq 4 was approximated by applying the trapezoidal rule to the measured concentration profiles for Li^+ and the hormones. The ratio of E_2 recovery to lithium recovery then was calculated by dividing the mass of E_2 recovered by the mass of lithium recovered. Because Q appears in both equations, the result is independent of the flowrate.

$$\frac{M_{E_2}}{M_{Li}} = \frac{\int_0^\infty C_{E_2}(t)dt}{\int_0^\infty C_{Li}(t)dt} \tag{5}$$

A similar approach was used for EE_2. Hormone recovery was assessed by comparing the observed hormone to lithium ratios (calculated using eq 5) to the ratios predicted from the mass of hormones and lithium added to the system (i.e., 5.4×10^{-4} for E_2 and 5.8×10^{-4} for EE_2). By this method, hormone recovery at mid-cell was approximately 52% for E_2 and 250% for EE_2. At the end weir, these values were 51% and 47%, respectively. When these values were corrected for hormone recovery (i.e., 80.2 ± 10.3% in spike recovery samples), recoveries were 65 and 310% at the midpoint and 64 and 59% at the end weir, respectively.

Effects of Sorption. Sorption to stationary surfaces, including bed sediments and vegetation, is the only process that is likely to retard the transport of hormones through the wetland. The hormones and lithium were added simultaneously to the well-mixed inlet weir of the test cell. As a result, if the hormones were not retarded, the shape of the hormone concentration profile would have been very similar to the lithium concentration profile, even if transformation had occurred. However, the hormone concentration profiles at the midpoint (Figures 3 and 4) and at the end (Figures 5 and 6) of the cell differed significantly from the lithium profile. In particular, the hormone profiles had multiple peaks, whereas a single major peak was observed in the lithium concentration profiles. Because the first major peaks in hormone concentrations observed at the end weir coincided with the lithium peak, it appears that a fraction of the hormones passed through the wetland without being retarded. The peaks that were observed after the lithium peak indicate that some fraction of the hormones added to the system underwent retardation.

At both sampling points, hormones were detected before the lithium tracer. At the midpoint, EE_2 and E_2 were detected at least 14 h before elevated concentrations of lithium were observed. At the end weir, EE_2 was detected 22 h before the lithium pulse appeared, and a relatively small E_2 peak was detected 20 h before the lithium pulse. Conservative tracers, such as lithium, should not undergo retardation in an engineered treatment wetland. Therefore, it is

surprising that hormones were detected before the lithium. Certain wetland sediments exhibit relatively high cation-exchange capacities (Stolt et al., 2000). However, it is unlikely that cation exchange was responsible for retardation of lithium in this system because monovalent cations generally do not undergo appreciable removal via cation exchange. Furthermore, the HRT calculated from the lithium residence time distribution was similar to the HRT calculated from the estimated volume and flowrate of the wetland cell. The HRT, which was estimated without adjusting for porosity (which would have increased the estimate), was 61 h, and the observed HRT was 85 h. The appearance of hormones before the lithium peak also does not appear to be attributable to sample contamination or another analytical artifact because hormones were never detected in the deionized water blanks and background samples that were analyzed throughout the study. Additionally, samples that had been contaminated with the stock solution would have had equal concentrations of E_2 and EE_2 because the stock solution used for spiking the wetland water contained approximately equal amounts of both compounds.

One possible explanation for the detection of hormones before lithium is that there was enhanced transport in the organic microlayer on the surface of the water. Previous research has shown that hydrophobic organic contaminants, such as the pesticide deltamethrin (log $K_{ow} = 6.2$), are sometimes enriched in the surface organic film (Muir et al., 1992). If hormone transport was higher in this organic matrix than in the water, it would be possible for a fraction of the hormones to move through the wetland faster than the lithium. However, there is no experimental evidence that this process occurs in wetlands, and further research would be required to test this theory.

Despite the unexpected detection of hormones before lithium, more than 90% of the mass of hormones recovered was detected during and after the lithium peak. At the midpoint, a pulse of hormones was observed that was approximately coincident with the major lithium peak. A hormone peak that coincided with the lithium peak also was observed at the end weir, but because of dispersion and attenuation, this peak was not as distinct. Hormone peaks also occurred after the lithium peak had passed. The observed hormone profile was inconsistent with sorption in a homogeneous system, where a single somewhat broadened peak would be expected, delayed relative to lithium. The observed results could be explained if water passed through the wetland in two distinct zones: a bottom layer in which sediments and biofilms on plant matter were prevalent and a surface layer with fewer surfaces available for sorption. According to this explanation, hormones in the upper layer of the water column would have had less opportunity to interact with surfaces than hormones in the lower water column. The hormones traveling in the upper part of the wetland would appear concurrently with the lithium, while hormones in the lower layer of the water would have been retarded relative to lithium. However, there are other potential explanations for the observed behavior, including density stratification in the water column, and further research is required to make any definitive conclusions. The observed concentration profile, with several peaks observed after the initial, unretarded peak, was consistent with previous studies on the fate of organic contaminants in wetlands. For example, when chlorpyrifos was added to the inlet of an engineered wetland, its concentration at the outlet exhibited periodic fluctuations similar to those observed in this wetland (Moore et al., 2002).

Effects of Biotransformation. At the end weir, the recoveries of E_2 and EE_2 were 64 and 59%, respectively, after correcting for recoveries. Hormone concentrations returned to their background levels after the pulse, indicating that the experiment continued for enough time to observe the entire hormone pulse. Furthermore, because abiotic processes, such as phototransformation, are not likely to be important in this densely vegetated wetland, bacterial transformation of the hormones is the most likely cause of the incomplete hormone recoveries. Several studies have demonstrated that E_2 can be degraded by bacteria typically present in municipal wastewater effluents and surface waters with half-lives ranging from several hours to approximately one week (Jurgens et al., 2002; Ternes et al., 1999a). Furthermore, experiments in columns packed with freshwater sediments have shown degradation half-lives on the order of 1 day for E_2 (Das et al., 2004). The overall HRT of the cell used in this experiment was approximately 3.5 days. Therefore, the observed E_2 attenuation rate was consistent with observations in surface waters documented previously.

In most previous studies, EE_2 is not as easily degraded as E_2 by bacteria present in activated sludge and in rivers. However, there are specific cases where their removals are comparable, such as in systems containing nitrifying bacteria (Vader et al., 2000). The current study was not designed to separate the effects of different microbial communities. Nevertheless, it is likely that nitrifiers were present in the wetland during the study period, and therefore it is conceivable that the EE_2 removal rate was higher than expected from previous literature because of their activity. Engineered wetlands are designed to remove nitrate by denitrification (Bachand and Horne 2000a; Mitsch et al., 2000), and denitrification occurs in this wetland efficiently (Table 2, Bachand and Horne 2000a). Although measurements of ammonia or organic nitrogen were not made during this study, nitrifying bacteria generally are present in the aerobic zone of engineered wetlands (Szogi and Hunt, 2001), and previous work in the Prado wetland has shown evidence of nitrification (Bachand and Horne, 2000a).

The high concentration of EE_2 detected at the midpoint suggests a problem with the midpoint data. Hormone enrichment in the surface organic layer, which would have led to samples that were unrepresentative of the entire water column, could explain these observations. Unlike the situation at the end of the cell, where well-mixed samples were collected from a weir, samples at the midpoint were collected by submerging a beaker in the wetland water. Although we attempted to collect a representative sample from approximately 0.2 m below the surface, it is possible that the surface layer was disturbed during the sampling process and was over-represented in the sample. Although the concentration of EE_2 in the solution added to the wetland (2.40 mg/L) was below the hormone's aqueous solubility (4.8 mg/L), the lithium in the solution created a high ionic strength environment that could have decreased hormone solubility. Even though EE_2 is only slightly less soluble than E_2 (13 mg/L) and the hormones were close to their aqueous solubility in the solution added to the wetland, it is conceivable that some EE_2 could have precipitated out of solution and migrated to the organic microlayer, while the E_2 remained in solution (Lee et al., 2003). This could explain the failure to observe elevated E_2 concentrations coinciding with the high levels of EE_2. Although the EE_2 data at the midpoint are difficult to explain, the concentration profile of E_2 at the midpoint and the profiles of both hormones at the end weir still suggest hormone attenuation in the wetland.

Further evidence of hormone biotransformation was provided by the measurements of E_1. During the period in which E_2 was detected, $E_1$was measured in a limited number of samples at concentrations ranging from 12 to 35 ng/L. Estrone was not detected in any of the samples that preceded or followed the hormone peaks. The E_1 concentrations that were coincident with the hormone

pulses are significantly higher than previous measurements in the Santa Ana River and in WWTPs that discharge to the river (i.e., 5 to 10 ng/L). Because of the analytical difficulties with the GC/MS/MS method that were discussed previously, the reported concentrations of E_1 probably underestimate the actual values and should be considered only in a qualitative sense. Nonetheless, the results demonstrated that incomplete recovery of E_2 was accompanied by detection of E_1 at levels exceeding previously observed background concentrations. This suggests that the mechanism of E_2 attenuation was biotransformation.

Implications for Other Wetlands and Surface Waters. Although only approximately half of the added E_2 and EE_2 were removed in the Prado test cells, engineered treatment wetlands show promise for providing a cost-effective means of removing estrogenic hormones from municipal wastewater and agricultural runoff. The results of this study and previous research on wetlands suggest several possibilities for achieving more efficient removal of hormones. One possible mechanism involves operating the wetland at longer HRTs. For example, the full-scale Prado treatment wetland is operated with a HRT of 7 to 10 days, whereas the test cell had a HRT of approximately 3.5 days. Assuming that hormone removal processes followed similar first-order kinetics, over 75% of the hormones could be removed by the wetland in the full-scale system. Another possibility is to increase the density of available surfaces, which are important to both sorption and biotransformation. This could be achieved by decreasing the depth of the cell so that less water flows above the roots and sediments or by using subsurface wetlands to increase the proportion of water infiltrating the sediments. There are numerous other factors that could affect the rate of hormone removal in engineered wetlands, including temperature, depth, loading of organic matter, and type and density of vegetation, which could not be explored in this study. This research could be complemented with a series of laboratory-scale experiments investigating the effects of the above-mentioned factors on sorption and biotransformation in simulated wetland conditions.

Despite the potential for improved hormone removal in the full-scale system, available data indicate that hormone concentrations in the effluent of the full-scale wetland are not different from those detected in the Santa Ana River or in the effluent of the upstream WWTPs (Table 1). For example, E_2concentrations up to 7.6 ng/L were detected in the outlet of the Prado treatment wetland during July 2000 (Table 1). It is unlikely that such concentrations would occur if the rate of hormone attenuation were equal to the rate observed in this study, because hormone concentrations in wastewater effluents rarely exceed 10 ng/L (Belfroid et al., 1999; Huang and Sedlak, 2001; Spengler et al., 2001; Ternes et al., 1999b). However, these test cells have considerably denser vegetation than the full-scale wetland cells. Because removal by sorption and biotransformation is likely to occur principally on biofilms coating plant surfaces, transformation rates in the full-scale system might be slower than those observed in the test cells.

Conclusions

- Despite certain analytical difficulties, it appears that the estrogenic hormones E_2 and EE_2 were attenuated during a reactive tracer experiment conducted in the Prado wetland test cells. Overall hormone removal ranged from 36 to 41%.
- It is likely that the observed attenuation of hormones occurred because of a combination of sorption to surfaces and biotransformation.
- Additional research is needed to identify factors that could enhance the removal of hormones in wetlands.

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