

QUANTIFICATION OF STEROID HORMONES WITH PHEROMONAL PROPERTIES IN MUNICIPAL WASTEWATER EFFLUENT

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Abstract—Many fish use steroid hormones as pheromones to initiate behavioral and physiological changes during spawning. To assess the occurrence of steroid hormones with pheromonal properties in the aquatic environment and to evaluate the possibility that municipal wastewater discharges contain compounds that could affect fish reproduction by interfering with pheromones, several estrogens, androgens, and progestins were quantified by gas chromatography/tandem mass spectroscopy in effluent samples from 12 municipal wastewater treatment plants. Samples also were analyzed from an engineered treatment wetland, three groundwater wells, and one reservoir. Estrogens (17 β -estradiol and estrone) were detected in wastewater effluent at maximum concentrations of 4 and 12 ng/L, respectively. Androgens (testosterone and androstenedione) were detected at concentrations as high as 6.1 and 4.5 ng/L, respectively, whereas the synthetic progestin medroxyprogesterone was detected at concentrations up to 15 ng/L. Data from an effluent-receiving engineered treatment wetland and shallow groundwater wells suggested that these compounds were not rapidly attenuated. The measured concentrations of steroids often exceeded olfactory detection thresholds at which fish detect these steroids, and in several cases, the steroid concentrations were comparable to levels at which pheromonal responses have been observed in fish.

Keywords—Estrogens Androgens Progestins Fish pheromones Engineered treatment wetland

INTRODUCTION

Following the initial report that exposure of fish to municipal wastewater effluents results in feminization [1], considerable attention has been focused on quantifying concentrations of estrogenic steroid hormones in municipal wastewater effluents and surface waters [2–13]. Because less evidence indicates that hormone axes aside from the estrogen system are disrupted by exposure to wastewater effluents, few studies have addressed other steroid hormones. However, androgens, estrogens, and progestins have been shown to affect reproductive physiology and behavior in many species of fish at extremely low concentrations [14–23]. As a result, fish reproduction, in addition to feminization by estrogens, could be affected by other steroid hormones in wastewater effluents through interference with pheromonal signaling.

Biologists who study chemoreception in fish classify waterborne compounds that elicit behavioral or biochemical responses as odorants or pheromones. A hormonal odorant is defined as a compound that has olfactory activity but for which pheromonal activity has yet to be demonstrated; a reproductive pheromone is a substance, or a mixture of substances, that is released by an individual and evokes in conspecifics a specific and adaptive reproductive response, the expression of which does not require specific learning [21].

Androgens such as testosterone and androstenedione elicit both odorant and pheromonal responses in fish at extremely low concentrations [14,17,21]. For example, precocious male Atlantic salmon (*Salmo salar*) parr exhibit an odorant response to testosterone at concentrations as low as 0.003 ng/L (10^{-14} M) [17]. Androstenedione also has been shown to act as a

pheromone in the goldfish (*Carassius auratus*) at picomolar concentrations [21].

Estrogens have been shown to be hormonal odorants and possible pheromones. For example, the round goby (*Neogobius melanostomus*) expressed odorant responses to five androgens and estrogens, including estrone [22]. Estrone elicited odorant responses in the goby at concentrations as low as 3 ng/L (10^{-11} M) and possible pheromonal responses, such as increased ventilation rate, at concentrations as low as 30 ng/L (10^{-10} M) [22].

Among the three classes of steroid hormones, progestins are particularly important to fish chemoreception. The best-studied progestin, 17,20 β -dihydroxy progesterone (17,20 β P), acts as a pheromone in many species of fish, often eliciting significant increases in blood steroid and gonadotropin hormone levels at extremely low concentrations [15,16,19–21]. For example, male goldfish are acutely sensitive to 17,20 β P, with a detection threshold as low as 0.03 ng/L (10^{-13} M) [15,16]. Pheromonal responses to 17,20 β P, including increases in milt volume, sperm motility, sperm quality, and spawning success, have been observed in male goldfish at concentrations as low as 3 ng/L (10^{-11} M) [24,25].

Although they have not been studied in detail, compounds that exhibit structural similarities to pheromones also can stimulate pheromonal responses [16]. In the goldfish, which has the best-studied pheromone system, a strong correlation has been reported between olfactory potency and structural similarity to 17,20 β P [16]. As a result, synthetic steroids used in human therapy could interfere with pheromone signaling in fish. In particular, those compounds exhibiting structural similarity to 17,20 β P, such as medroxyprogesterone, may act as potent fish pheromones.

A suite of steroid hormones likely is present in wastewater effluent at similar concentrations to the estrogens, because the

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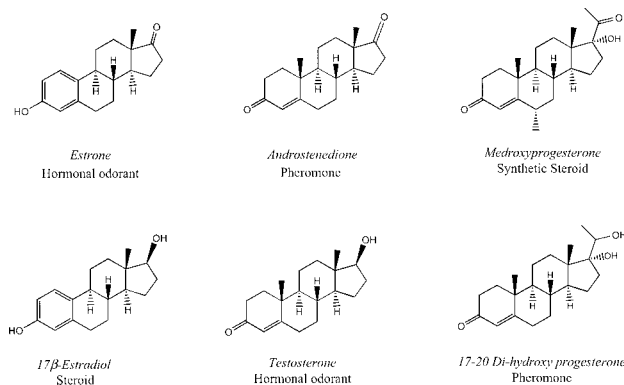


Fig. 1. Structure of steroids.

chemical properties that affect removal in wastewater treatment plants (e.g., hydrophobicity) and mass loading from human excretion are similar for most of the estrogens, androgens, and progestins. If concentrations of androgens and progestins in municipal wastewater effluent are as high as typical concentrations of estrogens (i.e., 0.1–20 ng/L [2–13]), then chemoreception by fish may be disrupted in effluent-receiving waters. Because pheromone-mediated reproductive processes result in behavioral and biochemical changes triggered by detection of the pheromone, disrupted chemoreception might lead to biochemical and behavioral changes at inopportune times. Responses to pheromonal cues at inappropriate times are believed to be energetically costly, can detract from feeding activities, and may expose fish to an increased risk of predation [24]. Furthermore, masking biologically important chemical signals through competitive binding to olfactory receptors has been suggested as a mode of action through which pollutants alter sensory perception [26].

To determine the prevalence of pheromonal steroids in the aquatic environment, a group of androgens, estrogens, and progestins (Fig. 1) were quantified in wastewater effluents and wastewater effluent-receiving surface waters. Comparison of the concentrations of pheromonal steroids in wastewater effluent with data from previous studies of chemoreception was used to assess the possibility that endpoints other than feminization are important to fish survival, especially where waste-

water effluent accounts for a significant fraction of a river's overall flow.

MATERIALS AND METHODS

All reagent chemicals and products were purchased from Fisher Scientific (Pittsburgh, PA, USA) at the highest possible purity (>98%). Steroids were purchased from Sigma-Aldrich (St. Louis, MO, USA), also at the highest possible purity (>98%). Aqueous solutions were prepared using water produced from a Nanopure II system (Barnstead, Dubuque, IA, USA).

Sample collection

All samples were collected in 12-L fluorinated Nalgene (Rochester, NY, USA) containers. Grab samples of wastewater effluent were collected from 12 municipal wastewater treatment plants (WWTPs) (Table 1). In all cases, grab samples were collected from the final effluent of the WWTPs, and additional grab samples from other locations (i.e., prechlorination) within these WWTPs were collected when possible (usually one or two grab samples per WWTP). Wastewater treatment plant 1 was sampled once during November 2001, May 2002, July 2002, and August 2002. Wastewater treatment plants 2 through 7 were sampled once during July 2002. Wastewater treatment plant 8 was sampled on 4 d of April 2001, on 4 d of August 2001, and once during July 2002. Wastewater treatment plants 9 through 12 were sampled once during November 2001, May 2002, and August 2002. Sample collection at these WWTPs was organized around a winter-spring-summer schedule when possible.

Municipal WWTPs 1 through 8 employed primary and secondary treatment, and WWTPs 9 through 12 employed additional tertiary treatment consisting of nitrification/denitrification with coagulation and mixed-media sand filtration. All but one of the plants used chlorine for disinfection. Whenever possible, samples were taken after effluent dechlorination. Because plant design precluded collection of dechlorinated samples in WWTPs 7 and 8, samples were collected before effluent chlorination. In November 2001, May 2002, and August 2002, samples were collected from three groundwater wells located in an urban area and one drinking-water reservoir. Two of the groundwater wells were equipped with multilevel samplers

Table 1. Description of municipal wastewater treatment plants (WWTPs)^a

WWTP	Flow (MGD [m ³ /s])	Primary treatment	OD/FP	Secondary treatment			Tertiary treatment		Cl ₂	UV disinfection
				AS	O ₂ AS	TF	NDN	MMF		
1	125 (5.5)	X		X					X	
2	70 (3.1)	X							X	
3	70 (3.1)	X							X	
4	17 (0.7)	X							X	
5	7 (0.3)	X		X					X	
6	2 (0.1)	X		X					X	
7	2 (0.1)	X							X	
8	2 (0.1)	X							X	
9	10 (0.4)	X		X			X	X	X	
10	1.5 (0.07)		X		X		X	X	X	
11	16 (0.7)		X		X	X	X	X	X	
12	1 (0.04)	X		X	X	X	X	X	X	X

^a MGD = million gallons per day; OD/FP = oxidation ditch/facultative pond; AS = activated sludge, air diffused; O₂ AS = activated sludge, pure oxygen; TF = trickling filter; NDN = nitrification/denitrification; MMF = mixed media filtration; Cl₂ = chlorine disinfectant; UV = ultraviolet light.

Table 2. Gas chromatography/tandem mass spectroscopy analytical conditions^a

Compound	Retention time (min)	Parent ion	Product ion ^b	Collision energy (eV)	LOD (ng/L)	LOQ (ng/L)
Mesterolone	19.10	414 (SIM)				
Hexachlorobenzene	6.76	284 (SIM)				
Testosterone	14.39	681	451, 466, 665	1.10	0.1	0.3
17 β -Estradiol	14.78	664	451	1.20	0.1	0.3
Androstenedione	15.88	482	253, 268, 467	1.00	0.1	0.3
Estrone	16.22	467	422, 448	1.00	0.2	0.4
Medroxyprogesterone	19.60	479	383	1.15	0.2	0.4

^a LOD = limit of detection; LOQ = limit of quantification; SIM = single-ion monitoring.

^b Tandem mass spectroscopy only.

screened in the deep and shallow aquifers. Grab samples also were collected from several locations in an engineered treatment wetland in April and August 2001. The engineered treatment wetland consisted of a series of interconnected ponds with the sole water source being the municipal wastewater effluent from WWTP 8. Based on the results of a separate tracer study that indicated a 6- to 7-d hydraulic retention time in the wetland, samples were collected from four locations throughout the wetland at 2-d intervals over a period of 8 d. All samples were placed on ice and transported to the laboratory, where they were extracted within 24 h of collection.

Sample solid-phase extraction and derivitization

To remove suspended particles, 4 L of sample were pressure-filtered through 90-mm AP-40 (Millipore, Bedford, MA, USA) glass-fiber filters. Although not measured for all samples, total suspended solids were generally less than 10 mg/L and were often much lower. The filtrate was collected, spiked to 100 ng/L with mesterolone as a surrogate standard, and pressure-extracted through 90-mm Empore (3-M, Minneapolis, MN, USA) C-18 solid-phase extraction discs. The synthetic steroid mesterolone was chosen as the surrogate standard, because it exhibits chemical properties similar to those of the other steroids and is not commonly used in human therapy. The C-18 discs were conditioned before use by rinsing twice with 25 ml of methanol followed by two rinses with 50 ml of distilled water. After extraction, the C-18 discs were rinsed twice with 25 ml of a 60:40 (v/v) water:methanol solution to selectively elute polar organic matter from the solid-phase extraction discs. After this wash step, steroids were eluted from the C-18 discs with 20 ml of a 25:75 (v/v) water:methanol solution. The eluent was completely dried under vacuum, resuspended in pure methanol, and transferred to a 1-ml volumetric flask. The extract was again dried under vacuum and resuspended in 200 μ l of acetonitrile. Next, 50 μ l of heptafluorobutyric anhydride (purity, >98%) were added as the derivatizing agent. The volumetric flasks were sealed and placed in a 55°C oven for 1.5 h. The samples were cooled to room temperature, and the solvent was evaporated under a gentle stream of nitrogen. The derivatized steroids were resuspended in 100 μ l of iso-octane to which hexachlorobenzene (400 μ g/L) had been added as an internal standard.

Steroid analysis

Steroid derivatives were analyzed by gas chromatography/tandem mass spectroscopy (GC/MS/MS; Thermoquest, San Jose, CA, USA). A 30-m \times 0.25-mm (inner diameter) \times 0.25- μ m (film thickness) MDN-5S column (Supelco, Bellefonte,

PA, USA) was used for separation. Splitless injections of 3.0 μ l into a 250°C injection port were used. Helium was used as the carrier gas at 1.2 ml/min. The programmed temperature run consisted of an initial 2.0-min hold at 80°C, followed by a 40°C/min ramp to 230°C with a 10.5-min hold, followed by a 3°C/min ramp to 240°C, followed by a 40°C/min ramp to 290°C with a 2 min hold. Mass spectrometer conditions included electron-impact ionization at 70 eV in a 230°C ion source with a 290°C transfer line from the gas chromatograph. Details of the retention times and mass spectrometer conditions are summarized in Table 2.

Sample quantification and quality control

Positive identification of steroids was based on retention time and MS/MS daughter-ion abundance ratios (Fig. 2). For analyte identification and quantification, retention times for the analytes had to match retention times of reference compounds within 0.1 min. Also, the abundance ratios of the MS/MS daughter ions had to match the abundance ratios of the reference compounds within 20%. Calibration was performed with linear, seven-point curves from 1.0 to 100 μ g/L, with points equally spaced on a logarithmic scale. The limit of quantification was based on the lowest calibration point of the calibration curve (i.e., 0.3 or 0.4 ng/L after accounting for sample preconcentration). The method limit of detection is approximately one-third of the limit of quantification (i.e., \sim 0.1 ng/L). Additionally, to be considered for quantification, the signal-to-noise ratios for the analytes needed to exceed six. Quantification was accomplished using the summed areas of the MS/MS base peak ion and any confirmatory qualifier ions. Peak areas were normalized to the surrogate standard (mesterolone) area count to correct for variations in derivitization efficiency, analyte recovery, and GC/MS/MS performance. The data also were normalized using the internal standard, hexachlorobenzene.

Quality assurance and quality control consisted of at least one distilled water blank, one duplicate sample, and one matrix recovery sample spiked at 10 ng/L with a mixture of testosterone, 17 β -estradiol, androstenedione, estrone, and medroxyprogesterone per 10 samples. Distilled water blanks, which were used to assess potential sample contamination, indicated that contamination never occurred. Duplicate samples, which were used to assess method reproducibility, always agreed within 10%. Recovery in spiked samples (18 total) was found to be 74.1% \pm 27.4% (mean \pm SE) and was correlated with the recovery of mesterolone. Little variation in recovery was observed among the five analytes, indicating similar loss mechanisms through the analytical method for these steroids.

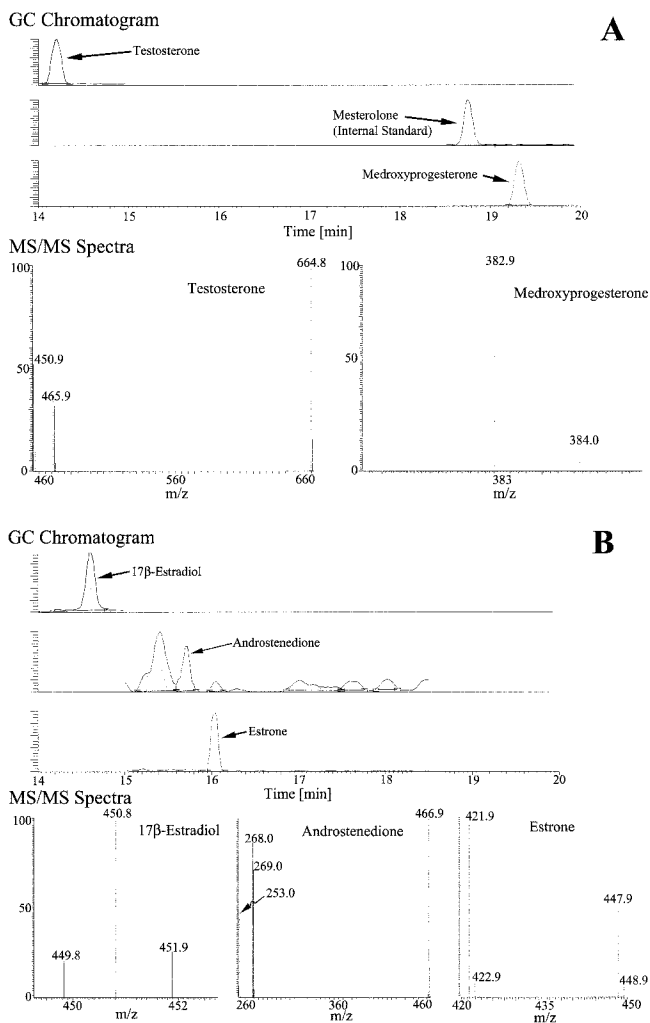


Fig. 2. Gas chromatography (GC) and tandem mass spectrometry (MS/MS) for (A) testosterone and medroxyprogesterone in wastewater treatment plant (WWTP) 1 effluent and (B) 17 β -estradiol, androstenedione, and estrone in WWTP 5 effluent.

Some positive interference, as evidenced by an increased response relative to that of the standards, was observed in certain environmental samples. This interference, presumably a result of organic matter matrix effects, was accounted for by the surrogate standard.

RESULTS

All five steroids were detected in samples from at least one of the eight secondary WWTPs (Fig. 3). The data in Figure 3 are from the July 2002 sampling survey; the samples are all from the final effluent of the WWTPs and are examples of effluents directly discharged into receiving waters. At least one steroid was detected in WWTP effluent at concentrations above the limit of quantification at six of the eight conventional WWTPs. The measured steroid concentrations covaried within treatment plants. For example, relatively high concentrations of four steroids were detected in effluent from WWTP 1, whereas no steroids were detected in any sample from WWTP 2 or 3. No obvious correlation was observed between steroid concentrations and WWTP capacity. With the exception of 17 β -estradiol in WWTP 4, steroids were not detected in effluent from secondary WWTPs equipped with pure oxygen-activated sludge processes (i.e., WWTPs 2–4). More sampling

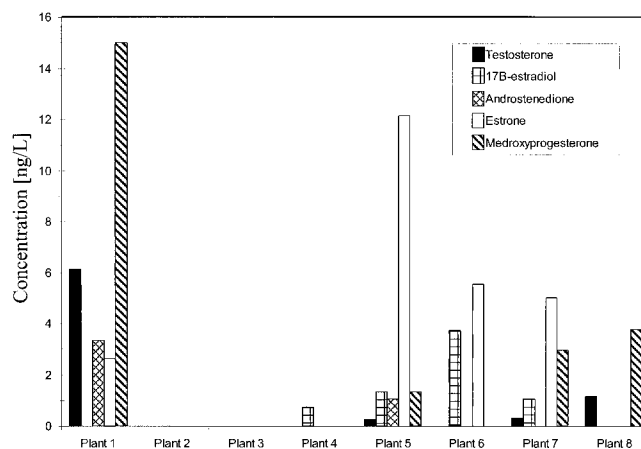


Fig. 3. Concentration of steroid hormones in municipal wastewater effluent samples.

would be needed to determine whether pure oxygen-activated sludge processes are particularly well suited for the removal of steroid hormones.

Samples from the four tertiary WWTPs (i.e., WWTPs 9–12) indicated good removal of steroids (data not shown). Among the 10 samples analyzed, steroids were only detected twice. Estrone was detected in one of the three effluent samples from WWTP 10 at 10.8 ng/L, whereas medroxyprogesterone was detected in one of the three effluent samples from WWTP 12 at a concentration below the limit of quantification.

Three groundwater monitoring wells located in an urban area were sampled during November 2001, June 2002, and August 2002. In November 2001, 17 β -estradiol and testosterone were detected in both the shallow and deep samples from one of the urban locations (data not shown). The 17 β -estradiol was present at 0.9 ng/L in the shallow sample and below the limit of quantification in the deep sample. For both samples in which 17 β -estradiol was detected, testosterone also was detected at concentrations below the limit of quantification. Estrone was present at 1.6 ng/L in another of the urban wells during June 2002. No steroids were detected in any of the other groundwater samples. Although the detection of steroids in groundwater samples was unexpected, other wastewater indicators (e.g., alkylphenol polyethoxylate detergent metabolites) were detected in each of the samples that contained hormones (J. Debroux, Kennedy/Jenks Consultants, San Francisco, CA, USA, personal communication), suggesting that these wells were affected by raw or treated wastewater. No steroids were detected in samples from the drinking-water reservoir.

To assess the fate of hormones in effluent-dominated surface waters, samples were analyzed from an engineered treatment wetland. One confounding factor that complicates the assessment of steroid attenuation in this wetland system is the large variability in steroid concentrations discharged by the associated WWTP, which vary by as much as an order of magnitude in consecutive 24-h composite samples. Previous sampling at this WWTP and others has demonstrated that large temporal variation of steroid concentrations in the final effluent occurs, presumably because of temporal variations in WWTP performance. Despite the variability in effluent composition, the measurements provided some insight regarding hormone fate in the treatment wetland and effluent-dominated surface waters exhibiting similar characteristics (e.g., high biological activity).

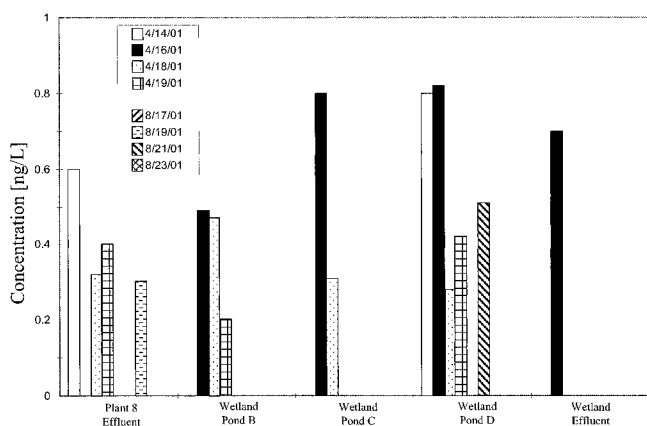


Fig. 4. Concentration of testosterone in samples collected from an engineered treatment wetland.

Testosterone was detected in 15 of the 40 samples from the engineered treatment wetland (Fig. 4). Concentrations of testosterone as high as 0.8 ng/L were observed in the wetland during April 2001. The concentrations of testosterone discharged by the WWTP during this period ranged from 0.3 ng/L (at the limit of quantification) to 0.6 ng/L, with testosterone being detected in three of the four effluent samples. Testosterone was detected only in 2 of the 20 samples collected during August 2001. During this period, the testosterone concentration in the WWTP effluent was 0.3 ng/L, whereas testosterone was detected in wetland pond D at 0.5 ng/L.

Estrone was detected in 34 of the 40 samples from the engineered treatment wetland (Fig. 5). Estrone concentrations ranged from 0.3 to 1.0 ng/L during April 2001 and from 2.1 to 12.3 ng/L during August 2001. During April 2001, estrone concentrations in the wetland ponds were comparable to concentrations in the WWTP effluent. During August 2001, estrone concentrations in the wetland were generally higher than those detected in the WWTP effluent.

Medroxyprogesterone and 17 β -estradiol also were detected in the wetland. Medroxyprogesterone was detected in 5 of the 40 wetland samples at concentrations ranging from 0.4 to 0.7 ng/L (data not shown). No pattern was evident in these data; medroxyprogesterone was observed in wetland ponds B and C during April 2001 and was observed in the wetland effluent during August 2001. The 17 β -estradiol was detected in 23 of the 40 samples at concentrations ranging from 0.3 to 4.1 ng/L. Details related to the fate of 17 β -estradiol in the engineered treatment wetland will be presented elsewhere. Androstenedione was not analyzed in any of the wetland samples.

DISCUSSION

The concentrations of 17 β -estradiol and estrone detected in municipal wastewater effluent were comparable to those reported previously [2–12]. The detection of androstenedione, testosterone, and medroxyprogesterone at concentrations comparable to those of 17 β -estradiol and estrone was consistent with predictions based on human excretion data [27] and similarities between the chemical properties that affect removal during wastewater treatment. The detection of medroxyprogesterone was consistent with estimates based on prescription data, which indicated concentrations of approximately 80 ng/L in raw sewage [www.rxlist.com [28]].

Although numerous studies have documented the occur-

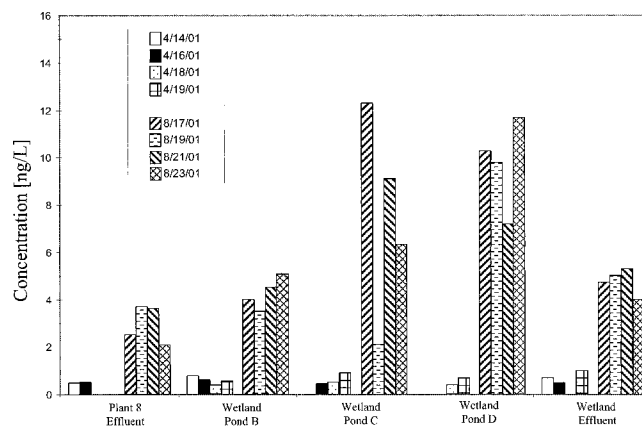


Fig. 5. Concentration of estrone in samples collected from an engineered treatment wetland.

rence of estrogens in wastewater effluent and surface waters [2–13], to our knowledge only three studies have included any data regarding androgens or progestins in the aquatic environment [13,29,30]. The U.S. Geological Survey (U.S. GS) reported the presence of testosterone and progesterone in numerous surface water samples as part of a comprehensive monitoring study [13]. However, the steroid hormone data in the U.S. GS study have been criticized, because the gas chromatography/mass spectroscopy (GC/MS) techniques employed are susceptible to artifacts from organic matter [31]. The GC/MS/MS technique employed in the present study avoided the problems associated with organic matter interference, because only the steroid hormones meet the stringent MS/MS criteria based on daughter-ion ratios. Another study, which implicated androstenedione in reproductive abnormalities in fish, indicated the presence of androstenedione in paper mill effluent at concentrations of approximately 40 ng/L [29]. Androstenedione also has been reported in primary municipal wastewater effluent at an estimated concentration of 105 ng/L [30]. However, the GC/MS method employed in that study is susceptible to many of the same artifacts as the U.S. GS study.

Although it is difficult to draw any conclusions regarding the efficacy of wastewater treatment processes in removing steroids without detailed studies of each treatment plant, it is reasonable to assume that conventional secondary WWTPs release low and variable concentrations of each of the steroid hormones considered in the present study. Furthermore, other endogenous and synthetic steroids also likely are present in wastewater effluent at similar concentrations (i.e., 0.1–20 ng/L). Data from tertiary treatment plants suggest that steroid concentrations can be reduced by installation of readily available tertiary treatment technologies.

Measurements of steroid concentration in the engineered treatment wetland indicate considerable variability between the two sampling events and, in several instances, concentrations of steroids within the wetland that are higher than those detected in the effluent of WWTP 8. The variability in testosterone and estrone concentrations is attributable to variations in WWTP performance rather than to sources of steroids within the wetland. In a related study, concentrations of 17 β -estradiol and ethinyl estradiol in 24-h composite samples of the final effluent of WWTP 8 varied by as much as an order of magnitude over a 10-d period (unpublished data). Although some estrone could have been produced by oxidative transformation of 17 β -estradiol in the wetland, the median 17 β -estradiol con-

Table 3. Measured concentrations, olfactory thresholds, and pheromonal effects levels for several steroids

Steroid	Species	Concentration (ng/L)	Reference	Measured concentration (ng/L)	
				Median	Range
Testosterone	<i>Salmo salar</i>	0.003 ^a	[17]	<LOQ ^c	<0.3–8.0
	<i>Petromyzon marinus</i>	3 ^b	[14]		
Androstenedione	<i>Carassius auratus</i>	0.3, 13 ^a	[21,38]	<LOQ	<0.3–4.5
	<i>Carassius auratus</i>	300 ^b	[23]		
Estrone	<i>Neogobius melanostomus</i>	3.0 ^a	[22]	2	<0.4–12.3
	<i>Neogobius melanostomus</i>	30 ^b	[22]		
17,20 β -dihydroxyprogesterone	<i>Carassius auratus</i>	0.03 ^a	[15]	NA ^d	NA
	<i>Carassius auratus</i>	3.0 ^b	[16]		
Medroxyprogesterone	? ^e	? ^e		0.3	<0.4–14.9

^a Olfactory detection threshold.

^b Concentration at which pheromonal effects have been demonstrated.

^c LOQ = limit of quantification.

^d NA = not analyzed.

^e Highlights a research need in the area of olfactory potency of synthetic steroids.

centration during the August 2001 sampling event was less than 1 ng/L, and less than 30% of the 17 β -estradiol was removed in the wetland (data not shown).

The measurements of steroid concentration in the engineered treatment wetland suggest that these steroids are not attenuated to a significant degree over a period of approximately one week (i.e., the hydraulic residence time of the wetland). Because this treatment wetland consisted of an interconnected series of shallow, vegetated ponds, it probably is a good surrogate for steroid attenuation in surface waters. The large surface area, high biological activity, and high particle concentration in this wetland system should approximate the most favorable conditions for removal of steroids in surface waters. As a result, the steroids in wastewater effluent likely could persist at concentrations similar to effluent concentrations until dilution occurs.

These results also support the premise that steroids originating in municipal wastewater effluent could affect fish reproduction by acting as pheromones. Available evidence suggests that many species of fish use steroid hormones or their conjugates as reproductive pheromones [14–23,32]. Concentrations of steroids in municipal wastewater effluent often exceed olfactory thresholds at which fish sense pheromones, and they sometimes exceed levels at which fish have been shown to respond to pheromones (Table 3). Furthermore, the current state of research on pheromone-mediated processes in fish is not exhaustive; pheromone responses may occur at even lower concentrations than those listed in Table 3 for more sensitive species of fish or during periods when fish demonstrate increased olfactory sensitivity (i.e., near spawning). As a result of the high sensitivity of fish to pheromones, physiological and behavioral endpoints related to pheromone-mediated reproductive processes may provide sensitive bioassays for determining whether exposure to wastewater effluent affects reproductive performance in fish [33,34].

The measurements of steroid concentration reported here suggest that wastewater discharges could disrupt pheromone signals in certain species of fish. For example, androstenedione is a pheromone that is primarily released by nonovulating female goldfish and is believed to inhibit biochemical responses to the pheromonal progestin 17,20 β P in male goldfish, preventing energetically costly biochemical responses at inopportune times [21]. If receptive male fish were spawning in an effluent-dominated river containing elevated levels of androstenedione, reproductive responses to 17,20 β P could be

inhibited in a manner similar to that caused by androstenedione-releasing females [21].

The detection of relatively high concentrations of the synthetic progestin medroxyprogesterone in wastewater effluent also raises the possibility that this synthetic steroid, or others like it, could interfere with fish reproduction by binding to olfactory receptors for the pheromone 17,20 β P. Previous research has demonstrated that a suite of progestins with structures similar to that of 17,20 β P are capable of mimicking 17,20 β P by eliciting gonadotropin hormone and milt responses at concentrations slightly higher than threshold concentrations for 17,20 β P [16,35]. Given the structural similarities between medroxyprogesterone and 17,20 β P, the competitive binding of medroxyprogesterone to olfactory receptors also could hinder 17,20 β P binding and reduce olfactory sensitivity to this pheromone, even if medroxyprogesterone is itself incapable of eliciting a biological response through olfactory receptor binding.

Although scientists have not studied population-level effects of chemicals that interfere with pheromone signaling, available evidence suggests that it could yield an adverse effect. For many species, pheromone-associated chemoreception is important during spawning, when male and female fish must coordinate egg and milt release [36,37]. Spawning requires a series of behavioral and physiological changes, some of which are initiated by chemical cues delivered by pheromones. In particular, ovulation (females) and milt release (males) require initiation of a complex series of biochemical cascades, and successful fertilization requires nearly simultaneous release of milt and eggs. Pheromones have evolved as the method that many fish species use to accomplish nearly simultaneous milt and egg release [19]. For example, in the male goldfish, 17,20 β P decreases feeding but increases locomotor activity, social interaction, aggression, sperm number, and sperm motility [24]. Together, these factors contribute to increased spawning success for goldfish exposed to 17,20 β P, as evidenced by the fact that pheromone-exposed male goldfish spawned with females two to five times as frequently as control males [24]. Pheromone-exposed male goldfish also exhibited increases in sperm quality and fertility that have led biologists to conclude that a response to 17,20 β P is a major determinant of reproductive success in male goldfish [25]. Given the importance of pheromones in the reproduction of fish, any interference with chemoreception from anthropogenic steroids during reproduction is likely to be detrimental to the long-term health of affected fish populations.

CONCLUSIONS

The steroid hormones androstenedione, testosterone, and medroxyprogesterone are present in municipal wastewater effluent at concentrations comparable to those of 17 β -estradiol and estrone. Once discharged to surface waters, these steroids are relatively stable, with little removal being observed in an engineered treatment wetland during periods of as long as one week. The concentrations of steroids in municipal wastewater effluent often exceed olfactory detection thresholds for pheromones and may be high enough to elicit pheromonal responses in certain species of fish. Although many factors influence the health of fish populations, interference with pheromone-mediated behaviors resulting from exposure to steroids in wastewater effluent could pose a previously unrecognized threat to the health of fish populations. Given the growing prevalence of effluent-dominated surface waters and the increased use of wastewater for aquatic habitat restoration, subtle changes in fish populations attributable to disruption of pheromone-based chemical communication should be considered. More research is needed to determine if exposing sensitive species to anthropogenic steroids with pheromonal properties adversely affects population fitness, especially in the case of rare and endangered species.

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