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Environmental Toxicology and Chemistry, Vol. 35, No. 11, pp. 2657–2664, 2016

Printed in the USA

Environmental Chemistry

PARENT AND CONJUGATED ESTROGENS AND PROGESTAGENS IN SURFACE WATER OF THE SANTA ANA RIVER: DETERMINATION, OCCURRENCE, AND RISK ASSESSMENT

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(Submitted 8 December 2015; Returned for Revision 4 February 2016; Accepted 30 March 2016)

Abstract: The present study investigated the occurrence of 13 parent and conjugated estrogens and progestagens in surface water of the Santa Ana River. With the exception of the synthetic hormones 17α-ethynylestradiol and mestranol, other compounds were detected at least twice at 10 representative sites, with the ubiquitous estrone (E1) and 17β-estradiol-3-sulfate as the dominant compounds quantified (0.24–6.37 ng/L and 0.49–9.25 ng/L, respectively). Sites near dairy farms exhibited high levels of conjugates, whereas those close to a sewage treatment plant (STP) effluent outlet displayed relatively high concentrations of E1. Principle component analysis coupled with multiple linear regression revealed dairy farms and the STP as the 2 significant contamination sources, accounting for 69.9% and 31.1% of the total hormone burden, respectively. Risk assessment results suggested E1 and 17β-estradiol (E2) as the 2 hormones with the largest risks to aquatic organisms, and which combined, contributed >90% of the total estrogenicity. Most of the sites investigated showed that E1 and E2 posed a medium risk (0.1 < risk quotient < 1), whereas each induced a high risk (risk quotient >1) at sites severely impacted by the STP and dairy farms. These results suggest that river health would benefit from effective treatment of waste at the STP and dairy farms prior to discharge. *Environ Toxicol Chem* 2016;35:2657–2664. © 2016 SETAC

Keywords: Hormones Occurrence Contamination sources Risk assessment

INTRODUCTION

Concern has been raised over the prevalence of steroid hormones (e.g., estrogens, progestogens, and androgens) in recent years due to their endocrine disrupting effects on aquatic organisms at levels as low as nanograms per liter [1,2]. The adverse effects include intersexuality, feminization, and reproductive and behavioral problems [3–6]. Steroid hormones are produced naturally in vertebrate bodies or synthesized by drug companies. The natural and synthetic hormones are also administrated considerably to humans and livestock for medical use. A significant portion of the hormones is excreted via urine and feces, mainly in the form of biologically inactive conjugates and partially as the active precursors or metabolites [7]. The latter causes significant environmental contamination [8].

Hormones enter the environment mainly through municipal wastewater treatment plant effluent and agricultural runoff after manure amendment [8]. The released hormones undergo a series of processes, such as biodegradation [9], photolysis [10], plant consumption [11], and sorption to soils [12], ultimately reaching environmental waters at nanograms per liter levels [13]. In recent decades, a handful of studies have assessed the presence of certain hormones and their site-specific risks in surface water [14,15] and groundwater [16,17]. In spite of this, the spatial distribution, environmental inputs, and co-occurrence of many compounds in different environmental compartments are still not fully understood.

Focusing our attention on Southern California, the Santa Ana River is the largest river in this region and plays a paramount role in supporting local biodiversity and freshwater supply. The flow of the river begins in the San Bernardino Mountains and extends to the Prado Basin and Santa Ana Canyon, passing through an area with 1 of the highest concentrations of cattle in the United States [18]. Concerns arise from the contamination of this river by endocrine disrupting compounds (hormones, etc.) released via urban or agricultural runoff due to the application of animal wastes or discharged directly from concentrated animal feeding operations and wastewater treatment plants. Previous studies have reported the occurrence of pathogenic *Escherichia coli* with antimicrobial resistance, pharmaceuticals, and alkylphenol ethoxylate metabolites in the Santa Ana River [18,19]. However, it is not known whether the hormones occur at concentrations sufficient to pose a threat to the aquatic organisms in the river. It is also unclear whether the major sources are municipal wastewater or animal feeding operations. To date, studies concerning the detection of hormones in the surface water of the Santa Ana River are rare.

The objectives of the present study were 3-fold: 1) to develop a liquid chromatography/mass spectrometry (LC/MS) method; 2) to investigate in the Santa Ana River the spatial occurrence of parent and conjugated estrogens and progestagens; and 3) to assess the potential risks posed to aquatic organisms. To assign the contamination sources and evaluate the contribution of the sources to total hormone burden, we performed principle component analysis followed by multiple linear regression, which has previously been applied for source apportionment of certain steroid hormones in river water [20] and for polycyclic aromatic hydrocarbons in sediment [21] and the atmosphere [22]. Such results are essential for pollution control and risk assessment in the river and its surrounding area.

This article includes online-only Supplemental Data.

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Published online 6 April 2016 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3447

MATERIALS AND METHODS

Chemicals and reagents

High purity standards of 8 steroids, including 4 natural estrogens (estriol [E3], estrone [E1], 17β-estradiol [E2], 17α-estradiol [α-E2]), 2 synthetic estrogens (17α-ethynylestradiol [EE2], mestranol) and 2 progestagens (progesterone and medroxyprogesterone) were purchased from Sigma Aldrich. The conjugated hormones, 17β-estradiol-17-glucuronide (E2-17G), 17β-estradiol-3-glucuronide sodium salt (E2-3G), estrone-3-glucuronide (E1-3G), 17β-estradiol-17-sulfate sodium salt (E2-17S) and 17β-estradiol-3-sulfate sodium salt (E2-3S), were obtained from Steraloids. Purity of all compounds was ≥98%. American Chemical Society (ASC)-grade sulfuric acid was bought from Mallinckrodt Chemicals. The ACS-grade ammonium hydroxide solution (NH_4OH; 28.8%, v/v) was purchased from Mallinckrodt. Acetonitrile and methanol of high performance liquid chromatographic (HPLC) grade were obtained from Burdick & Jackson. Acetic acid, ethyl acetate, and HPLC-grade water were purchased from Fisher Scientific. The abbreviations and general chemical information can be retrieved from Supplemental Data, Table S1. The chemical structures are shown in Supplemental Data, Figure S1.

An individual standard stock solution (1000 μg/mL) was prepared by dissolving 10 mg powder of each compound in 10 mL methanol and stored at –20 °C for a maximum of 6 mo. Twice per week, a 10 μg/mL intermediate composite of all compounds was prepared by combining the individual standard stock solutions. A working solution was prepared daily by diluting the previous solution with 20% methanol.

Site selection and sample collection

The Santa Ana River is approximately 150 km long and samples were taken from an area covering an approximate 40 km length of the river. The study area and sampling sites are shown in Figure 1. Ten representative sites along the river and its major tributary were selected, including areas impacted by animal feeding operations (S1, S2, S3, and S4), gradients upstream or downstream of sewage treatment plants (STPs; S6, S7, S8, and S9), and sites that near residential or industrial areas (S5 and S10). Further information on the sampling sites is provided in Supplemental Data, Table S2.

From August 31 to September 3, 2015, grab samples of the surface water were collected in 1 L amber glass bottles. Duplicate samples were taken at each site. The pH was adjusted to 2 onsite using condensed sulfuric acid to minimize microbial activity. The addition of sulfuric acid has been shown to be a better method to preserve parent and conjugated hormones than formalin and $HgCl_2$ over the pH range of 1.1 to 2.5 [23]. Water samples were kept in a cooler with ice during transport to the laboratory and processed within 24 h of arrival. River water samples collected at the surface of the river (34°01′26″ N, 117°21′52″ W) during fine weather in August 2015 were employed for analytical method development.

Sample extraction and cleanup

River water was filtered through Whatman glass fiber filters (pore size, 0.7 μm; GE Life Sciences) to prevent cartridge clogging. For the extraction and purification of target compounds, an aliquot of 500 mL of the filtered river water was loaded onto solid phase extraction (SPE) cartridges on a 12-port Superco vacuum manifold connected to a vacuum system. Waters Oasis hydrophilic–lipophilic balance (HLB) cartridges (200 mg, 6 mL) were preconditioned using 6 mL methanol followed by 6 mL water. Water samples were passed though the SPE cartridge at a flow rate of 3 mL/min to 5 mL/min. The sampling bottles were rinsed twice with distilled water, which was subsequently loaded onto the cartridges. After the water samples were loaded, the cartridges were rinsed sequentially with 10 mL methanol:water (5:95, v/v); 10 mL water solution containing 5% methanol and 2% acetic acid (by volume); and 10 mL water solution containing 5% methanol and 2% NH_4OH (by volume) [24]. Prior to elution, the cartridges were dried under vacuum for 45 min. The target compounds were eluted by passing 4 mL of ethyl acetate:methanol (90:10, v/v) followed by 4 mL methanol containing 2% NH_4OH (by volume) though the cartridges. The eluents were collected in graduated glass tubes and evaporated under a gentle nitrogen stream at 40 °C in a water bath until approximately 0.2 mL volume was achieved. This was then brought to a final volume of 0.5 mL with 20% methanol. The reconstituted extracts were filtered through 0.22 μm Teflon membrane filters (Fisher Scientific) for instrument analysis.

Instrument analysis

An Agilent 1100 HPLC coupled with a 6410 triple quadrupole MS/MS system with an electrospray ionization (ESI) source was employed to analyze the target compounds. The chromatographic separation was performed on an Agilent ZORBAX Extend-C18 (3.0 mm × 150 mm, 3.5 μm) column adaptable to a high pH (3–11) mobile phase. The injection volume was 20 μL. The flow rate was 0.35 mL/min, and the

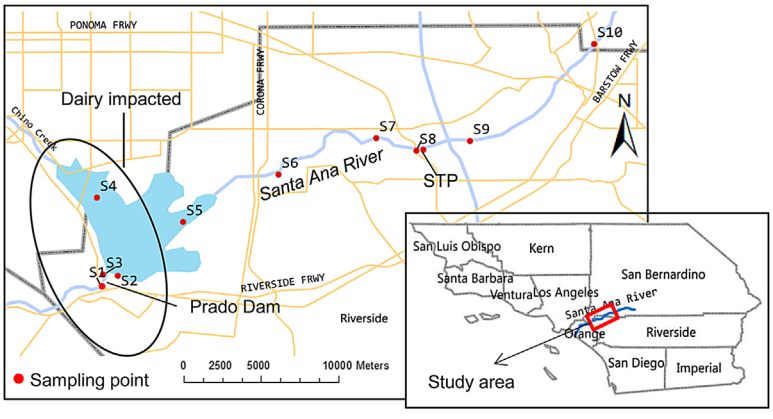

Figure 1. Map of sampling sites.

temperature was kept constant at 40 °C The mobile phase was composed of (A) 0.15% (v/v, pH ~9) NH_4OH in water and (B) acetonitrile:methanol (80:20, v/v), and the optimum gradient was programmed as follows: 12% B from 0 min to 2 min, increasing to 50% B at 10 min, 95% B at 15 min, and then hold for 5 min before restoring to the initial composition at 20.5 min, with the final composition held for 7.5 min. The total run time was 28 min. Mass Hunter software Ver B.01.04 was used for data acquisition, processing, and instrument control. The mass spectrometer was operated at either the negative (ESI–) or positive mode (ESI+) depending on the compound properties. High purity nitrogen gas was used as a drying, nebulizing, and collision gas. The conditions for the mass spectrometer were as follows: gas temperature, 325 °C; dry gas flow, 12 L/min; nebulizer, 50 psi; and capillary voltage, 4000 V (+) and 3500 V (–). For each compound, the characteristic product ions of protonated $[M+H]^+$, and deprotonated precursors $[M-H]^-$ or $[M-Na]^-$ were monitored in positive and negative mode, respectively (Supplemental Data, Table S1). Each precursor/product pair composed a multiple reaction monitoring transition. The more abundant transition was used as a quantifier and the other as a qualifier. Time segment monitoring was used to maximize the intensity. Multiple reaction monitoring optimized parameters, including precursor ions, product ions, fragment energy and collision energy, are given in Supplemental Data, Table S1.

Method validation and quality control

Matrix effects were evaluated by spiking a mixture of standard solution containing 25 ng of each compound into 500 mL river water extract and were calculated by dividing the peak area obtained at the fortification level (after subtracting the peak area corresponding to the native analytes present in the extract) by that of the standard solution containing the same concentration of analyte and converting to a percentage (matrix effect %). A matrix effect % of less than or greater than 100% indicates ion suppression or enhancement, respectively. The recovery was assessed by spiking a low (2.5 ng) and high (25 ng) amount of each compound to 500 mL of river water. The reproducibility ($n=3$) at each spiking level was determined by percentage relative standard deviation (SD %). Instrumental detection limit was determined by direct injection of decreasing amounts of calibration standard down to 0.01 ng/mL. The instrumental detection limit was defined as the concentration level that produces a signal-to-noise ratio of 3. The method detection limit and method qualification limit were defined as the concentrations that produce signal-to-noise ratios of 3 and 10, respectively, based on the chromatogram of actual sample matrices at approximately 10× method detection limit spiked level. The identification was carried out by comparing the retention time (within 2%) and the ratio (within 20%) of the quantifier to qualifier. A single-point calibration method derived from a 5 ng/mL matrix-matched standard was carried out for quantification. The final concentration of the target compound in real samples was estimated by multiplying 5 ng/mL by the area ratio of the compound in real samples to that in matrix-matched standard solution (after background subtraction), as well as by multiplying the dilution factor 0.001 (derived by dividing the final constituted volume [0.5 mL] by the initial volume of water sample [500 mL]). For quality control, 1 procedural blank of distilled water and 1 matrix blank fortified with a known concentration of a mixture of target compounds were pretreated in each batch simultaneously with river water samples to check for background contamination and to validate the purification. Solvent blanks were inserted at the beginning and before river water samples in each sequence to minimize cross contamination from the previous injection.

Estrogenic activity and risk assessment

Potential risks of estrogenic activity to aquatic organisms were judged based on total estrogenic activity expressed as total estradiol equivalents (EEQ_{total}) and the risk quotient. The calculated EEQ_{total} was determined from the following equation [14]

$$EEQ_{total}\ (ng/L) = \sum EEQ_i = \sum (C_i \times EEF_i) \qquad (1)$$

where EEQ_i is the EEQ of a target compound i, C_i is the detected concentration of compound i, and EEF_i (the estrogenic equivalent factor) is defined as the ratio of the observed half-maximal (EC50) estrogenic activity of E2 to each compound. The EEF values were 0.47 for E1, 1 for E2, 0.029 for α-E2, 7.6×10^{-3} for E3, and 1.25 for EE2, respectively, which were derived from yeast estrogen screen (YES) assay results of previous studies [25,26]. Risk quotient is expressed as the ratio of measured environmental concentration (MEC) to the predicted no effect concentration (PNEC) of an individual compound, as described in the following equation

$$\text{Risk quotient} = MEC/PNEC \qquad (2)$$

where PNEC can be estimated by the method of species sensitivity distribution [14]. The toxicity data was acquired from the US Environmental Protection Agency's ECOTOX database. In the present study, we defined the PNEC values of each compound using the calculated 5th percentile value (HC5) from Nie et al. [14], where HC5 was acquired by applying the method of species sensitivity distribution, meaning that 95% of the species would not exhibit any adverse estrogenic effect.

Statistics

Varimax-rotated principle component analysis was performed to identify the most likely sources of parent and conjugated estrogens and progesagens in the water matrix. Multiple linear regression based on the principle components was adopted to interpret the contribution of major sources to the total hormone burden. The statistics were performed using SPSS software.

RESULTS AND DISCUSSION

Analytical procedure and method performance

A large amount of potential interferences, such as humic substances, were simultaneously retained on the Oasis HLB sorbent with the target compounds. Removing interferences is an essential step to reduce matrix effects. The initial SPE rinse with methanol:water (5:95, v/v) washed weakly bonded impurities off the cartridges. Basic interferences were reduced by washing with solution containing 5% methanol and 2% acetic acid, whereas the solution containing 5% methanol and 2% NH_4OH removed relatively hydrophilic acidic compounds, leaving acidic compounds that were relatively more hydrophobic, including steroid conjugates, on the column [23,24]. The nonpolar solvent ethyl acetate ensured the elution of parent estrogens and progestagens. The polar solvent methanol containing 2% NH_4OH allowed for the efficient elution of hydrophilic steroid conjugates and the less lipophilic compound E3 [27].

The hydrophilic steroid conjugates were retained less strongly on the HLB materials and eluted from the analytical column earlier than parent hormones (Supplemental Data, Figure S2). All the conjugates were determined in the negative ionization mode due to their acidic chemical nature. The chromatographic separation of the parent hormones allows sufficient time for mode switch, which enables the simultaneous acquisition of multiple reaction monitoring data in both modes during a single run (Supplemental Data, Figure S2). Because the intensity of 1 compound generally decreased with the increasing number of transitions being scanned simultaneously, we separated the whole detection window to 5 time segments: 4 min to 6.5 min (E2-17G), 6.5 min to 10.5 min (E2-3G, E1-3G, E2-17S, and E2-3S), 10.5 min to 13.5 min (E3), 13.5 min to 17.2 min (E2, α-E2, EE2, and E1), and 17.2 min to 20 min (medroxyprogesterone, progesterone, and mestranol). Compounds with the same 2 transitions, E2-17G and E2-3G for example, were divided into separate time segments. It is worth noting that the conjugates were more prone than parent hormones to be influenced by ionization suppression due to the isobaric interferences of hydrophobic acidic impurities that share similar properties. Improvement in chromatographic separation of the conjugates and impurities via gradient and temperature adjustment could be a rapid approach for attenuating such matrix effects. In the present study, temperature adjustment did not result in dramatic changes in the capacity factors of hormone conjugates based on the existing gradient. Indeed, reducing the initial mobile phase composition, for example from 20% to 12% B, to postpone the retention time of the conjugates could reduce the ionization suppression effect, while having minimal influence on the retention of parent hormones.

The addition of NH_4OH solution facilitates the deprotonation of conjugates. The sensitivity of parent and conjugated hormones was enhanced by NH_4OH compared with certain acidic or triethylamine additives and acetate buffer [27,28]. Previous studies have shown that methanol is superior to acetonitrile in terms of signal intensity enhancement but not in terms of the resolution of target estrogens, possibly because the higher viscosity of methanol favors ionization but hinders resolution [28]. Therefore, a combination of acetonitrile and methanol (80:20, v/v) was used for the organic mobile phase as a compromise between sensitivity and separation.

The optimized multiple reaction monitoring transitions and fragment and collision energies are shown in Supplemental Data, Table S1. The precursors for compounds detected in the negative and positive mode were in the form of $[M-H]^-$/$[M-Na]^-$ and $[M+H]^+$, respectively. The major fragmentation of the glucuronide conjugates featured the neutral loss of the glucuronide moiety, that is, $[M–H–C_6O_6H_8]^-$ [29]. The glucuronide anion was further cleaved to form an intense ion at m/z 113 [30]. In terms of sulfate conjugates, the quantifier with m/z 97 corresponding to $[HSO_4]^-$ formed when the sulfate was connected previously to the alicyclic ring (E2-17S). However, when the sulfate was attached to the aromatic ring (E2-3S), a more abundant peak at m/z 271.2 was produced resulting from the neutral loss of SO_3 ($[M–H–SO_3]^-$), because the formation of HSO_4^-, in the case of E2-3S, would form a benzyne, which is energetically prohibitive [23,31–33]. The major fragment ions at m/z 143, 145, 171, and 183 for estrogens, resulting from ring cleavage, were ascribed to $[C_{10}H_7O]^-$, $[C_{10}H_9O]^-$, $[C_{12}H_{11}O]^-$, and $[C_{13}H_{11}O]^-$ respectively [34]. The quantitative product ions of progestagen at m/z 109, 123, and 97 corresponded to $[C_7H_9O]^+$, $[C_9H_{15}]^+$, and $[C_6H_9O]^+$, respectively [35,36].

The instrumental detection limit ranged from 0.01 ppb to 1 ppb (Supplemental Data, Table S1), estimated at a signal-to-noise ratio of 3. The method performance parameters are presented in Supplemental Data, Table S3. Except for mestranol, the recoveries at low- and high fortification levels were all above 60%, which could be ascribed to the distinct ionization suppression of the hydrophobic neutral compounds. Except for mestranol, the values of matrix % were all less than 100% but greater than 70%, suggesting a low ionization suppression in the present methods. The reproducibility, represented as relative SD % for recoveries and matrix %, were generally within 30%. Throughout the whole procedure, contamination from blanks was not observed as indicated by the distilled water blanks. Figure S3 (Supplemental Data) shows the multiple reaction monitoring chromatograms of detected compounds in a river water sample from the sampling site S2.

Occurrence of parent and conjugated estrogens and progestagens

The occurrence and distribution of each compound are shown in Figure 2 and Supplemental Data, Table S4. All target compounds except mestranol were detected at least once in the river water. The ubiquitous detection of E1 (100% frequency) was generally in line with previous observations in environmental waters [8,27,37]. As for the conjugates, sulfate conjugates were generally more frequently detected than glucuronide conjugates because of the recalcitrant nature of sulfate conjugates due to weaker arylsulfatase activity of *E. coli* compared with β-glucuronidase during the wastewater treatment process [38,39]. The E2-3S was detected with higher frequency (100%) in the Santa Ana River than in other rivers [27,40]. The frequency of E3 (20%) was similar to the result presented for riverine water of Guangzhou [37], but lower than results reported for other countries [16,41]. The frequency disparity suggests that the detection of some compounds may display site-dependent variations.

The natural estrogens E1 (0.24–6.37 ng/L), E2 (0–0.84 ng/L), and E3 (0–0.7 ng/L) were present in the Santa Ana River at concentrations comparable with the range reported in Iberian rivers [42], the Cache La Poudre River [43], Japanese rivers [27], and in surface waters of the State of Delaware [15], but were lower than the presented results in rivers of China [44]. Alpha-E2, a form of estrogen excreted by cattle, but not by humans or other livestock [45], is usually detected in large concentrations in dairy wastes (∼730 ng/L) [46]. In the present

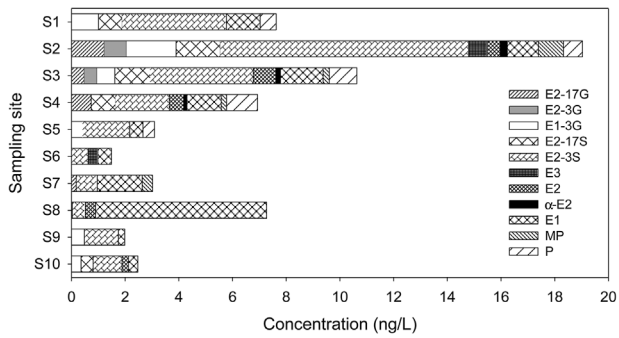

Figure 2. Concentrations (ng/L) of parent and conjugated estrogens and progestagens in surface waters of 10 sampling sites. Results were reported as averages of duplicate values. E2-17G = 17β-estradiol-17-glucuronide; E2-3G = 17β-estradiol-3-glucuronide; E1-3G = estrone-3-glucuronide; E2-17S = 17β-estradiol-17-sulfate; E2-3S = 17β-estradiol-3-sulfate; E3 = estriol; E2 = 17β-estradiol; α-E2 = 17α-estradiol; EE2 = 17α-ethynylestradiol; E1 = estrone; MP = medroxyprogesterone; P = progesterone.

study, it is not surprising that α-E2 was only detected at sites near dairy farms (S1–S4) and was not expected to be present frequently at other sites. Lower concentrations of α-E2 in the environmental water compared with the livestock wastewater were partially ascribed to the conformation transformation to its β-isomer and the microbial-mediated oxidization to E1 [47–49]. Conjugated estrogens were found in previous investigations to constitute up to 22% of total estrogen loads from dairy farming [46]. Therefore, it would be more logical to find quantifiable amounts of conjugated hormones at sites S1 through S4, which are close to dairy farms. The average concentrations of E2-3S ranged from 0.49 ng/L to 9.25 ng/L, with the highest value present at the upstream reservoir of Prado Dam (S2). Progesterone was detected at 5 of the 10 sites, with the concentrations as high as 1.15 ng/L, similar to the results presented in the Llobregat river basin in Spain [50] and rivers in China [51].

The synthetic estrogens mestranol and EE2, common human oral contraceptives, were the least frequently detected compounds. The absence of mestranol is reasonable, because mestranol is rapidly transformed to EE2 after administration [52], and mestranol was also absent from river water in other studies [37,52]. The detection of EE2 in relation may be influenced by how frequently the population served by the STP used it and the treatment process the STP used. The low excretion of EE2 to total estrogens in the US (∼<1%) [53], combined with its high removal rate during sewage treatment [54], possibly accounts for the occurrence of EE2 with levels 1 order of magnitude lower than E1 in the water receiving STP discharges [55,56]. The predicted level of EE2 occurring in STP effluents was in the range 0.13 ng/L to 0.76 ng/L [57], lower than the quantification limit achieved in the present study (Supplemental Data, Table S3). The synthetic progestagen medroxyprogesterone is used widely as human or veterinary medicine for contraception, treating endometriosis, and hormone replacement therapy [58,59]. In the present study, medroxyprogesterone was detected at S2, S3, S4 (near dairy farms) and S7 (downstream from the STP) at concentrations 0.2 ng/L to 0.93 ng/L (Figure 2), comparable with the range observed in other dairy impacted waters [60]. Site S7 is adjacent to a golf course and several households that kept a small number of horses. The detection of medroxyprogesterone in the surface water downstream from the STP (S7) but not upstream (S9) suggests that the synthetic progestagen was possibly used for veterinary purposes. Ruling out variations in the analytical method performance for these synthetic compounds, demographic data and their removal efficiency by the STP thus likely explain the relatively low detection frequencies and low concentrations.

A higher concentration of E1 (6.37 ng/L) was detected at S8 (where the STP effluent and river water combined), whereas only 0.24 ng/L of E1 was detected upstream of the STP (S9); this suggests that the STP effluent may be the major source of E1. Estrone was reported by myriad studies to be the major steroid in STP effluents partially because of the high bacteria-mediated cleavage of E1 conjugates or the transformation of E2 [52,61]. Although E1 occurred in high concentrations at S8, it was attenuated quickly as the distance downstream increased. At S6, only 0.51 ng/L of E1 was detected, which is close to the upstream concentration. With the exception of the sulfate conjugate E2-3S, which is relatively resistant to bacterial degradation, concentrations of other compounds declined as the distance downstream of the STP increased (Supplemental Data, Table S4), consistent with previous observations [52]. Labadie and Budzinski [52] reported estrone at not detectable concentrations in suspended particulate matter, suggesting that sorption was not the major sink for E1 in the Jalle d'Eysines River, which received 9% to 33% of its flow from the STP effluent. Results presented by Holthaus et al. [62] showed that <1% sorption occurred when assessing the removal of E2 and EE2 in rivers with silty sediment and rich particulate organic carbon. Therefore, biodegradation was, in our present research, possibly the major pathway of decay of these compounds considering the sandy streambed and the extremely low suspended particulate matter content of the Santa Ana River. The porous nature of the sandy soil limits its sorption potential but favors infiltration and subsequent biodegradation of hormones [15].

Source interpretation

The samples were collected from sites that were potentially contaminated. The principle component analysis results revealed 2 principle components after varimax rotation, accounting for 55.27% and 17.41% of total variance, respectively (Table 1). The first component was highly associated with estrogen conjugates and progestagen, whereas the second component was highly correlated with E2 and E1, indicative of the source related to dairy farms and the STP, respectively. The conjugated estrogens and E1, with the former the dominant form excreted in dairy waste if untreated [46,63] and the latter the most abundant estrogen in STP effluent after treatment [64,65], provided support for the 2 major contamination sources. Multiple linear regression was performed using the principle component score matrix (t_k) obtained from principle component analysis as the independent variable and the total concentration of parent and conjugated hormones after standardization ($\widehat{Z}_{sum}$) as the independent variable, to give a model written as

$$\widehat{Z}_{sum} = 0.851t1 + 0.366t2 \; (r^2 = 0.859, \; p < 0.001) \qquad (3)$$

where $\widehat{Z}_{sum}$ is the total concentration of target parent hormones and hormone conjugates; $t1$ and $t2$ are interpreted as the sources, that is, dairy farms and the STP, respectively. The values 0.851

Table 1. Varimax-rotated component matrix and source contribution obtained from principle component analysis and multiple linear regression[a]

	Component[b]	
Variable	1	2
17 β-estradiol-3-sulfate (E2-3S)	.949	
17 β-estradiol-17-sulfate (E2-17S)	.917	
17 β-estradiol-3-glucuronide (E2-3G)	.893	
Estrone-3-glucuronide (E1-3G)	.784	–.386
17 β-estradiol-17-glucuronide (E2-17G)	.763	
Progesterone (P)	.700	.313
Medroxyprogesterone (MP)	.673	
17 β-estradiol (E2)	.523	.780
Estrone (E1)		.775
Percentage variance explained (%)	55.3	17.4
Possible source	Dairy runoff	Sewage treatment plant
Contribution (%)	69.9	30.1

[a]Eigenvalue cutoff 1.0; rotation method: Varimax with Kaiser Normalization.
[b]Absolute value >0.3 are shown.

and 0.366 are the coefficients (B_k) of the 2 components in the regression equation. The multiple linear regression results demonstrated the first 2 components were both significant at the 0.05 level. The percentage contribution of each source to the total, defined as $B_k/\Sigma B_k$, was 69.9% for $t1$ and 30.1% for $t2$. The results indicated that a dairy associated source assumed 69.9% of the total detected hormone burden. Relatively, a small number of compounds were determined at low concentrations at S5, which was located close to a high density residential area, suggesting that no untreated domestic sewage was discharged directly in this area.

Risk assessment

Throughout the sampling sites, the total estrogenicity, expressed as EEQ_{total}, was lower than 2 ng/L, with the exception of S8, where average value of slightly higher than 3 ng/L was found (Figure 3). Young et al. [66] proposed a long-term E2 PNEC for freshwater life of 1 ng/L. The average EEQ_{total} in the present study surpassed the threshold concentration of 1 ng/L and would thus be expected to cause endocrine disruption in fish. Four of the 10 sites displayed an EEQ_{total} value larger than the threshold (Figure 3). The 4 sites were those that directly received effluent from the STP (S8) or were impacted by dairy farms (S2, S3, and S4). The overall total estrogenicity was within the range reported in Iberian rivers of Spain [42] and dairy impacted headwater streams in the United Kingdom [67]. Estrogen E1 and E2 contributed more than 90% of the total estrogenicity, consistent with previous results [67]. Compared with other studies in which the primary estrogenicity was induced by E2 and EE2 [14,42], we did not detect EE2 in the majority of samples. Nevertheless, this does not necessarily indicate that no risk was posed by EE2 in the Santa Ana River, because the detection limit (0.5 ng/L) in the present method was much higher than the EE2 PNEC (0.1 ng/L) [66]. Lowering the detection limit of EE2 could possibly indicate estrogenicity.

Another approach to evaluate the potential risks is via the risk quotient approach. A risk quotient value of <0.1 is considered riskless, <1 medium risk, and >1 high risk [68]. Compounds such as mestranol, EE2, E3, and α-E2 were not considered when calculating the risk quotient because of low concentrations or inadequate toxicity data. Considering the largest contributions of E2 and E1 to EEQ_{total}, only risk quotient values of E2 and E1 were reported at the 10 sampling sites, as indicated in Figure 4. In surface water, the majority of sites exhibited medium risk for most of the sensitive aquatic organisms in the Santa Ana River. Spatially, S3, impacted by dairy farms, and S8, receiving the STP effluent, displayed high risk (risk quotient >1). Generally, E2 posed relatively higher risk in dairy and industry impacted sites, whereas for E1 it was the STP-impacted sites. The risk quotient values were overall consistent with the EEQ results. Overall, E2 and E1 are considered the priority compounds of concern in the Santa Ana River. However, it is noteworthy that not all the compounds were taken into account when calculating EEQ_{total} or risk quotient. For example, α-E2 and EE2, with the latter being 1 of the most potent estrogen compounds with a PNEC as low as 0.1 ng/L, as well as the conjugates, which posed potential risk once transformed to their active parent forms [46], were not included. The present study provided a snapshot of the presence of the selected parent steroid hormones and conjugates. Hormone levels are subject to seasonal alteration, with the concentrations in summer potentially lower than winter resulting from high microbial biodegradation rates and intense photolysis in summer [52,69]; furthermore, rainfall may affect contamination during wet periods. The risk in the present study, therefore, may be underestimated based on the hormone concentrations occurring in late summer.

CONCLUSIONS

The present study reported a sensitive analytical method for the simultaneous determination of 13 parent and conjugated hormones occurring in the Santa Ana River. Estrone and E2-3S were the most abundant compounds (0.24–6.37 ng/L and 0.49–9.25 ng/L, respectively) with a 100% detection frequency. Estrogen conjugates were commonly detected in sites close to dairy farms. Source apportionment results revealed 2 significant sources, that is, dairy farms and the STP, which contributed 69.9% and 30.1% of the total hormone burden, respectively. Risk assessment outcomes suggested E1 and E2 were major compounds worthy of attention in the Santa Ana River, which when combined, contributed $>90\%$ of the total estrogenicity. Most of the examined sites displayed medium risks posed by E1 and E2. Sites close to dairy farms and the STP posed the highest threat to aquatic organisms in the Santa Ana River. This implies that dairy farms and the STP need to adopt

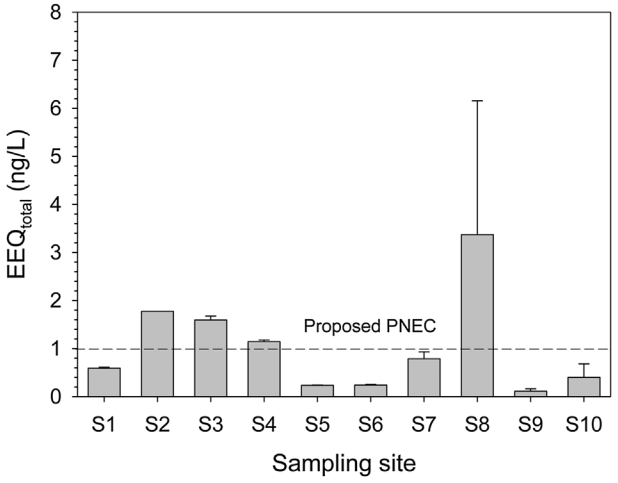

Figure 3. Average total estradiol equivalents (EEQ_{total}) calculated in river waters. The proposed predicted no effect concentration (PNEC) of estradiol is shown as a horizontal dash line. Error bars indicate standard deviation ($n = 2$).

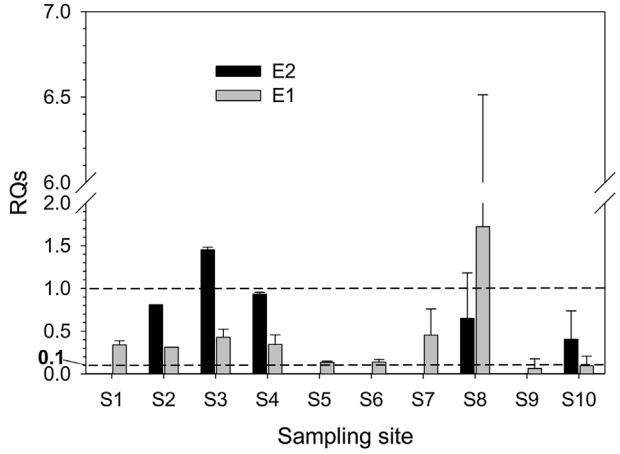

Figure 4. The calculated average risk quotients (RQs) of estrone (E1) and estradiol (E2) detected in water samples from 10 sites. Error bars indicate standard deviation ($n = 2$).

improved waste treatment measures before waste disposal. Future work will focus on methods of remediation of these compounds.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc. 3447.

Data availability—Supporting data are available on request; please contact our Contaminant Fate and Transport research group at US Department of Agriculture's Agricultural Research Service Salinity Laboratory, Riverside (Scott.Yates@ars.usda.gov).

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