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Analysis of Steroid Hormones in a Typical Dairy Waste Disposal System

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The environmental loading of steroid hormones contained in dairy wastes may cause an adverse effect on aquatic species. To better assess the potential risks of hormone contamination resulting from land application of dairy wastes, various steroid hormones were determined in a typical dairy waste disposal system. Quantitative methods using gas chromatography/mass spectrometry (GC/MS) were developed to monitor low levels of steroid hormones in complex solid and liquid samples contaminated with dairy manure. The preparation method for wastewater analysis consisted of solid-phase extraction and purification steps, which minimized interference from the sample matrices and achieved low detection limits for the studied hormones. In the dairy wastewater and lagoon water, three endogenous hormones—17α-estradiol, 17β-estradiol, and estrone—were detected. The concentration of 17α-estradiol in fresh milk parlor effluent rapidly decreased along the wastewater disposal route, whereas the concentration of estrone increased along this same pathway. This suggests that 17α-estradiol was readily oxidized to the metabolite estrone. Levels of total steroid hormones in the sequencing lagoon water were approximately 1−3 orders of magnitude lower than those in the fresh dairy wastewaters, indicating significant removal of these hormones during the transport of dairy wastewater from source to field. In solid dairy waste samples, four steroid hormones were identified and quantified. Increasing the piling time of solid wastes and increasing the residence time of wastewater in sequencing lagoons are suggested to be economical and efficient agriculture practices to extend the degradation time of hormone contaminants and thereby reduce the hormone load to the environment.

Introduction

The occurrence of steroid hormones in the aquatic environment is receiving considerable attention due to the fact that most of these compounds are classified as highly potent endocrine-disrupting chemicals (EDCs) (*1–3*). EDCs may interfere with the normal function of the endocrine system of humans and animals. Steroid hormones of general concern include estrogens (e.g., estradiol), androgens (e.g., testosterone), and gestagens (e.g., progesterone), which are either excreted endogenously from living creatures or are used as pharmaceuticals in human and veterinary clinical practices. These steroid hormones have been implicated as potential environmental contaminants because exposure to these chemicals, even at levels as low as nanograms-per-liter (ng/L), can adversely affect reproduction in a wide variety of aquatic species (*4–6*).

Two important sources of hormone contaminants to the environment are municipal sewage treatment plants (STPs) and concentrated animal feeding operations (CAFOs). Due to incomplete removal in most conventional biological STPs, natural and synthetic hormones, as well as their metabolites, ranging from <0.1 to 10 ng/L have frequently been detected in effluents of STPs and receiving surface waters (*7–10*). Recent research indicates that animal agriculture, like CAFOs, may be another major source of these contaminants to the aquatic environment (*11*). The livestock excretion (e.g., urine and feces) usually contains a large amount of natural and synthetic chemicals including hormones and antibiotics (*12, 13*). Unlike STPs, CAFOs do not require additional treatments as long as livestock wastes are not discharged directly into water bodies. However, land application of livestock wastes may result in the loading of hormones and their metabolites to agricultural fields, which may subsequently enter the aquatic environment by leaching and/or runoff (*14, 15*). This practice may therefore increase the occurrence of hormones in the environment and elevate their concentrations in surrounding watersheds.

Dairy farms are one of the most important CAFOs. A review by Lange et al. (*16*) calculated hormone excretion for various livestock species. They estimated that pregnant and cycling cows contribute about 90% of the endogenous hormones in the United States. To minimize the potential hormone inputs resulting from dairy wastes, the proper treatment and disposal of solid manure and manure-containing wastewater are critically important. Information pertaining to the transport and fate of hormones and their metabolites in the dairy waste disposal system, however, is still poorly documented. It is unclear what amount of hormones may be introduced into fields by agricultural practices, and whether the dairy wastes pose risks to surrounding aquatic environments due to the loading of hormone contaminants when dairy wastes are applied to fields at agronomic rates.

Low hormone concentrations and complex matrices have largely limited extensive surveys on the occurrence and abundance of steroid hormones in the environment. Most analytical methods to date focused on determining steroid hormones in sewage treatment effluents and receiving waterways (*2, 7–10, 17–19*). The enzyme-linked immunoassay technique is a convenient and sensitive method for the analysis of hormones (*20, 21*). However, the method lacks sufficient selectivity and also is prone to interferences caused by the presence of extract matrices and similar nontarget compounds (*20, 21*). Alternatively, gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) has frequently been adopted to accurately quantify concentrations of hormones. Methods based on GC/MS and GC/MS/MS require that the hormones be derivatized prior to analysis. Silylation and acetylation are two frequently applied derivatization techniques for steroid hormones and their metabolites (*22*). GC/MS/MS and LC/MS/MS have superior sensitivity and are highly selective techniques for ultratrace analysis of hormones. Their detection limits are generally well below 1 ng/L, which is appropriate to determine

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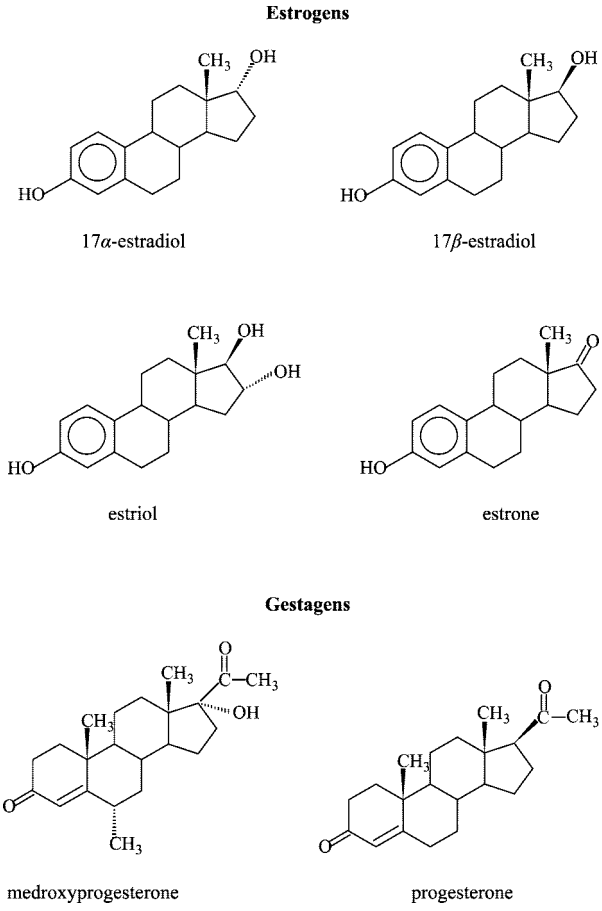

FIGURE 1. Structures of six steroid hormones of concern.

hormone concentrations in most surface water or groundwater samples where the levels of steroid hormones lie in the picograms-per-liter (pg/L) to ng/L range (*1, 19*). GC/MS and LC/MS can offer modest sensitivity for hormone analysis, and their detection limits are typically above ng/L-level (*7, 17, 23*). The concentrations of hormones in livestock wastes are relatively high and are typically 100−1000-fold higher than those in effluents of STPs and environmental samples. Hence, GC or LC with single MS has sufficiently low detection limits to identify and quantify hormones in dairy wastes (*20, 21, 23*).

The objectives of this study were (i) to develop a feasible, accurate, and reliable method to detect low levels of steroid hormones in complex matrices involving liquid and solid manure; and (ii) to investigate and evaluate the concentrations of hormones in a representative dairy waste disposal system in California. The target steroid hormones included four estrogens and two gestagens (Figure 1), which have frequently been detected from CAFO lagoon water (*1, 11*). The study conducted will be useful in understanding the types and amounts of steroid hormones in dairy wastes, as well as in addressing potential environmental issues related to the input of hormones after land application of dairy wastes.

Experimental Section

Chemicals. Internal standard hexachlorobenzene (99%) and hormone standards including 17α-estradiol, 17β-estradiol, estriol, estrone, medroxyprogesterone, and progesterone at the highest possible purity (>98%) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Stock solutions of each hormone were prepared in acetone. The derivatizing reagent heptafluorobutyric anhydride (HFBA), Carbograph solid phase extraction (SPE) columns, and Florisil SPE columns were purchased from Supelco (Bellefonte, PA). Other reagent chemicals were obtained from Fisher Scientific (Fair Lawn, NJ). All chemical reagents were used as received.

Field Sites and Sampling. All samples were collected from a dairy farm located in San Jacinto, California. This dairy has around 2,000 dairy cows including 1,000 milking cows. Typically, dairy solid manure is scraped mechanically from barns or feeding stalls using bucket loaders. As collected, the solid manure is composed of feces and urine with undigested feed residues and bedding materials. The collected solid wastes are piled together and dried before use as a soil amendment. Most of the dairy wastewater is discharged from the milking parlor, where cows are washed and the parlor is flushed before being milked. The wastewater runs freely by gravity to large waste lagoons via a sewage lane, with several slotted dams to remove coarse solids. The waste lagoon water is applied to surrounding fields for production of forage crops, when needed. This is a common nutrient recycling system on many California dairy farms. A diagram of the dairy waste disposal system is provided in the Supporting Information (Figure S1).

The fresh manure (<2 h after deposition) was taken from six sampling locations within barns. At each of these locations, an approximate 500-g sample was collected by means of an aluminum scoop, and then all samples were combined as the fresh manure sample studied. Piled manure was collected from three drying manure heaps. The age of the piled manure was around two weeks estimated through conversation with the operator of the facility. At each of these three heaps, 1000-g samples were randomly collected from the top 15 cm of a stack in several different locations and then combined together. Fresh dairy manure-containing wastewater (FW) was taken from the sewage outlet of the milking parlor after hydraulic flushing. Three dairy wastewater (DW I, DW II, and DW III) samples in the sewage lane were sequentially collected below the three slotted dams. Meanwhile, the solid wastes were sampled from the entrance of each slotted dam using a shovel. Three large lagoons were sequentially connected and the wastewater sample was taken adjacent to the outlet of each lagoon at the about 15 cm depth below the surface using a self-made stainless steel bucket. Additional information concerning the dairy waste disposal system and sampling sites is provided in the Supporting Information. All solid samples were placed in sealed plastic bags and liquid samples were stored in 4-L solvent bottles and immediately transported to laboratory in an ice cooler. Liquid samples were immediately extracted. Solid samples were placed in a freezer at −21 °C before analysis.

Hormone Analysis in Dairy Wastewater and Lagoon Water. Prior to extraction, 35 mL of dairy manure-containing wastewater (FW, DW I, DW II, and DW III) or 250 mL of lagoon water, respectively, was centrifuged to minimize plugging of the solid-phase extraction (SPE) cartridge. Briefly, each liquid sample was measured into Teflon centrifuge tubes and centrifuged at 12,000 rpm for 20 min. The clarified supernatant was decanted. The centrifuge solid was extracted with 10 mL of methanol for 1 h using a reciprocating shaker. After centrifuging at 5000 rpm for 15 min, the methanol extract was added to the previous aqueous supernatant and mixed thoroughly. The extraction was repeated two additional times. To inhibit the microbial degradation of hormones, formaldehyde (1% final w/v) was introduced to all samples. In this study, twelve replicates of each sample were performed at one time. To determine the recovery, six replicates of each sample were spiked with 1 ng/μL 17α-estradiol, 17β-estradiol, estriol, estrone, medroxyprogesterone, and progesterone standards (50 ng each).

Steroid hormones were extracted using Carbograph solid-phase extraction (SPE) according to a modified method described by Hanselman et al. (*23*). Before introducing the

liquid samples into the Carbograph SPE columns, the cartridge was sequentially washed with 10 mL of 80:20 (v/v) dichloromethane/methanol, followed by 5 mL of methanol acidified with 10 mM formic acid, 5 mL of methanol, and 10 mL of deionized water. The samples were passed through Carbograph SPE columns with the aid of vacuum to control the flow rate at ~1 drop/s. After the sample flowed through the cartridge, the SPE column was rinsed sequentially with distilled water, 15 mL of methanol acidified with 10 mM formic acid, and 5 mL of methanol. The estrogens were eluted with 10 mL of 80:20 (v/v) dichloromethane/methanol. The captured eluants were evaporated to dryness using a gentle nitrogen stream at 60 °C. The extracted residues were dissolved in 1 mL of 50:50 (v/v) dichloromethane/hexane.

To reduce the matrix background and purify the sample, a cleanup procedure was performed using Florisil gel (*2*). The Florisil SPE columns were preconditioned with 5 mL of 80:20 (v/v) dichloromethane/methanol, followed by 5 mL of 50:50 (v/v) dichloromethane/hexane. After the extracted samples were passed through the Florisil gel column, the cartridge was rinsed with 10 mL of 50:50 (v/v) dichloromethane/hexane and then eluted with 6 mL 80:20 (v/v) dichloromethane/methanol. The eluant samples were taken to dryness under nitrogen at 60 °C in preparation for derivatization.

Acetylation with HFBA was used to derivatize steroid hormones in the study. A preliminary experiment was performed to optimize the derivatization. Briefly, 50 or 100 ng of six hormone standards was dissolved in 200 μL of acetonitrile in a glass vial with a PTFE coated cap. All sample vials were sealed immediately after adding 50 μL of HFBA. The samples were derivatized under different temperatures (55 and 80 °C) with different derivatization time. After derivatization, the samples were cooled to room temperature and the solutions were evaporated under a gentle stream of nitrogen. The derivatized products were dissolved in 100 μL of hexane with hexachlorobenzene (100 ng/mL) as an internal standard and then analyzed by GC/MS.

On the basis of optimized derivatization results, the purified dairy samples were dissolved with 200 μL of acteonitrile, and then 50 μL of HFBA was added. The samples were incubated at 80 ± 1.0 °C for 1.5 h. The derivatized samples were cooled and evaporated under nitrogen prior to being redissolved in 500 μL (for dairy wastewater samples) or 100 μL (for lagoon water samples) of hexane that contained hexachlorobenzene (100 ng/mL) as an internal standard. Derivatized samples were subjected to GC/MS analysis.

Hormone Analysis in Dairy Solid Wastes. Extraction of steroid hormones in solid samples was performed using a modification of the method described by Raman et al. (*20, 21*). Briefly, a freeze-dried solid waste (2.5 g of fresh and piled manure, or 5.0 g of settled manure) was weighed into a 40-mL polyethylene centrifuge tube along with 5 mL of 1.0 M NaOH. All samples were immediately extracted by adding 10 mL of ethyl ether to minimize microbial degradation of the target analytes. The centrifuge tubes were vigorously shaken for 1 h and vortexed for 2 min at room temperature (21 ± 0.5 °C). Next, the samples were centrifuged at 5000 rpm for 15 min. The organic phase was transferred into a 50-mL flask, and the extraction was repeated two additional times. Extracts from each sample were combined together and then mixed thoroughly. The solvent was evaporated under a gentle steam of nitrogen, and 10 mL of acetonitrile was added to each flask to dissolve the analytes while the solution was swirled. The sample extracts were capped and stored at −20 °C.

Positive interference probably caused by the organic matter matrices was observed as an increased response in the sample extracts relative to that of the standards. Moreover, different steroid hormones display different interference effects. To account for the issue, solid samples were quantified by a method of standard addition. Briefly, extracts (1.0 mL) of each sample were spiked with standard solutions containing different concentrations of six hormones ranging from 5.0 to 200 ng/mL. All samples were taken to dryness under nitrogen. The dry samples were derivatized for hormone analysis as described above. The derivatized products were dissolved in 1.0 mL of hexane with internal standard hexachlorobenzene (100 ng/mL) and then analyzed by GC/MS. The calibration was performed with a six-point curve for each analyte with $R^2 \geq 0.990$.

GC/MS Analysis. A Hewlett-Packard (HP) 6890 GC in tandem with an Agilent 5975 mass selective detector (MSD) equipped with a HP-5MS column (30 m × 0.25 mm i.d. × 0.25-μm film thickness, J&W, Folsom, CA) was used for identification and quantitation of steroid hormones. The GC conditions were 1.2 mL min^{-1} carrier gas flow rate (He), 260 °C inlet temperature, and 290 °C interface temperature. The initial oven temperature was 80 °C for 2 min; the temperature was increased to 230 at 5.0 °C/min and held for 3.0 min, and then increased to 280 at 25 °C/min and held for 2 min. The electron energy and electron multiplier voltage were 70 eV and 400 V above autotune value, respectively. The detector was tuned each day by using perfluorotributylamine and the standard spectra autotune program.

Mass spectrometric analysis was carried out using both full scan and selected ion monitoring (SIM) modes. In the full scan mode, the electron impact (EI) mass spectra were generated using an electron energy of 70 eV and ions with m/z 200–900 were monitored. The full scan mode was used for the identification of analytes by fragmentation patterns and retention time as compared to known standards. For quantitation of hormones in all sample extracts, SIM mode was used. The following quantitation and confirmation ions were used in the SIM: $m/z = 284$ for hexachlorobenzene, $m/z = 664$ and 451 for 17α-estradiol and 17β-estradiol, $m/z = 876$ and 449 for estriol, $m/z = 466$ and 442 for estrone, $m/z = 479$ for medroxyprogesterone, and $m/z = 510$ for progesterone.

Each hormone in wastewater samples was quantified by a calibration standard curve containing seven points ($R^2 \geq 0.995$). Like each sample, calibration standards were derivatized and then analyzed with the internal standard. For each type of liquid and solid samples, quality assurance and quality control consisted of at least one water blank or one manure blank, one spike-blank, and one triplicate sample. Triplicate samples agreed within 10%, and hormones were never detected in any water blanks or manure blanks during the study. Method performance including the recoveries and limits of quantification (LOQs) of steroid hormones in the each type of sample is provided in the Supporting Information.

Results and Discussion

Optimization of Derivatization and GC/MS Analysis. Acetylation with HFBA is an excellent derivatization technique for steroid hormones, which yields highly informative fragment ions that allow definitive identification and quantification of analytes (*24*). The use of acetonitrile as a solvent to dissolve steroid hormones followed by HFBA derivatization achieved high yields without forming byproducts. This derivatization has been frequently used to quantify hormones in aquatic samples (*1, 10*). In the beginning of this study, we optimized the derivatization conditions for analysis of steroid hormones contained in dairy wastes. The results demonstrated that the derivatization temperature was of great importance for reproducibility. For eight replicate experiments with 50 ng and 100 ng of each analyte, coefficients of variation for the yields of the heptafluorobutyric (HFB) derivatives were less than 15% when the derivatization temperature was set at 80 °C (Table S1, Supporting Information). However, the use of

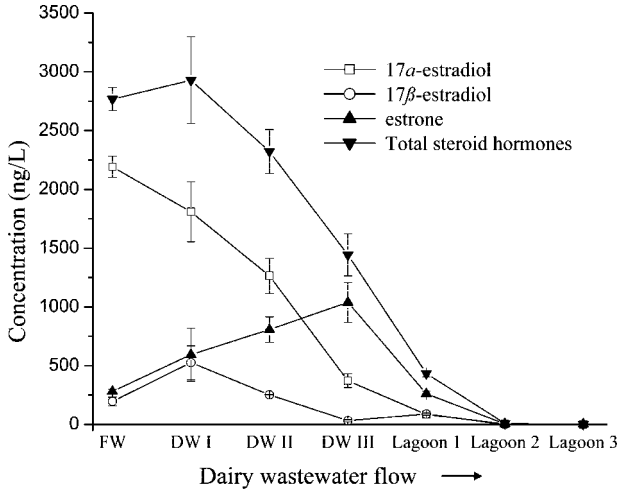

FIGURE 2. Concentration trends of steroid hormones along dairy wastewater flow. Abbreviations are as described in the text. Data shown are mean with standard deviation from six samples for each location that was sampled twice (November 2006 and March 2007).

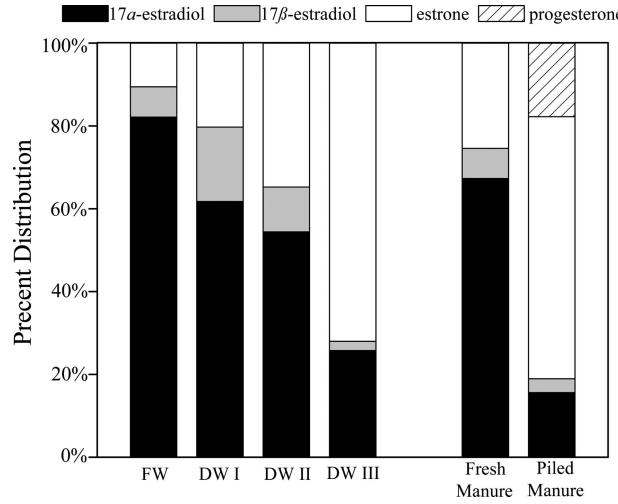

FIGURE 3. Percent distribution of each steroid hormone for total hormone equivalents. Abbreviations are as described in the text ($n = 6$ for each type of sample).

relatively low derivatization temperature (e.g., 55 °C used in refs (*1*) and (*10*)) led to poor method precision (RSD > 20%) in the quantitative analysis of steroid hormones (Table S1). It is similar to a previous report that indicates HFBA derivatization produced variable and low recoveries for hormone analysis in urine samples while the derivatization temperature was conducted at 60 °C (*25*). For all dairy samples in this study, the analytes were derivatized at 80 °C for 1.5 h. Further experiments indicated that increasing derivatization times and higher temperatures did not improve yield.

A representative selected ion chromatogram (SIM) obtained from a dairy wastewater sample is shown in Figure S2 along with its SIM spiked with six hormone standards. The chromatogram represents the typical retention times of the HFB derivatives of six hormones and typical matrix background for dairy wastewater samples. All HFB derivatives were baseline resolved, which indicates the matrix interference was well suppressed using the sample preparation methodology. The typical mass spectra of HFB derivatives of the studied hormones are shown in Figure S3 (Supporting Information), which implies that every hydroxyl group in the four estrogens (Figure 1) would react with HFBA.

Hormonal Profile of Dairy Wastewater and Lagoon Water. In the dairy wastewater and waste lagoon water, three estrogens (17α-estradiol, 17β-estradiol, and estrone) of the six steroid hormones investigated were detected, while estriol, medroxyprogesterone, and progesterone were never detected in any of the dairy liquid samples. The concentrations of estrogens in the dairy liquid samples are shown in Figure 2 and Figure S4.

In fresh dairy wastewater, the level of 17α-estradiol was almost an order of magnitude higher than that of 17β-estradiol or estrone (Figure S4). This reflects the initial composition of estrogens excreted by cattle, in that 17α-estradiol is much more prevalent than 17β-estradiol (*12, 27*). Conversely, the concentration of estrone was much higher than that of 17α-estradiol in the samples collected from DW III adjacent to the inlet of lagoon 1 (Figure S4). As illustrated in Figure 3, the percentage of 17α-estradiol of the total hormones accounts for >80% in FW sample and decreases to <30% in DW III sample. In contrast, the percentage of estrone increased to >70% in DW III from about 10% in FW. Previous research has demonstrated that estrone is one of main metabolites of 17α-estradiol (*11*). The concentration of 17α-estradiol decreased gradually along the disposal pathway of dairy wastewater, which was accompanied by a rise in the concentration of estrone (Figure 2). These results indicate that 17α-estradiol may be readily oxidized to estrone by manure microorganisms in dairy wastewater. This transformation is similar to the degradation of 17β-estradiol in sewage sludge (*27*) and swine sow lagoons (*28*). Noticeably, the transformation processes of both estradiol isomers to estrogen are indicated to be a reversible mechanism under anaerobic conditions (*11*). Therefore, reversible transformations can produce 17β-estradiol from estrone which was originally produced from 17α-estradiol. This racemization exhibits some potential for environmental impact, because it may alter the biological activity of dairy wastes in that 17β-estradiol is considered as a more potent EDC than 17α-estradiol (*29*).

The levels of total hormones in the lagoon water were much lower than those in the dairy wastewater (Figure 2). The large attenuation of hormones in lagoon water may be attributable to dilution, biodegradation, photodegradation, sorption, residence time, and settling of hormone-associated manure particles. This observation suggests that the environmental load of natural hormones in lagoon effluent is likely to be much less than that for direct discharge of fresh dairy wastewater. For the three sequencing lagoons, the total concentration of steroid hormones in the secondary and tertiary lagoons was less than 10 and 5 ng/L, respectively, which was almost 2 orders of magnitude less than that in the primary lagoon (Figure 2). Obviously, land application of dairy wastewater from the secondary or tertiary dairy lagoon rather than primary lagoon could minimize the hormone load to the environment. This observation may be primarily attributed to a longer residence time of dairy wastewater in the secondary and tertiary lagoons than that in the primary lagoon (see the Supporting Information), which allows more time to remove hormones by degradation (biodegradation, photodegradation, etc.) and settle hormone-associated manure particles. Therefore, using sequencing lagoons may be a simple and efficient practice to remove hormone contaminants. Also, additional research is needed to better understand and accurately determine the primary mechanisms that are responsible for lowering total hormone concentrations in these sequencing lagoons.

Hormonal Profile of Dairy Solid Manure Waste. Four of the six steroid hormones of interest were detected in the dairy fresh manure and piled manure. The concentration levels of hormones ranged from below the LOQ to 10^3 μg/kg (Table 1). On a dry basis, the 17α-etradiol accounted for the largest percentage of the total hormone in the fresh manure,

TABLE 1. Mean Dry Basis Abundance of Steroid Hormones in Dairy Solid Wastes[a]

	μg/kg				
compound	fresh manure (<2 h)[b]	piled manure (2 weeks)	dam 1 (3 months)	dam 2 (3 months)	dam 3 (3 months)
17α-estradiol	1416 ± 104	172 ± 9	43 ± 15	54 ± 14	8 ± 2
17β-estradiol	153 ± 25	37 ± 3	18 ± 4	<LOQ	N.D.[c]
estriol	N.D.	N.D.	N.D.	N.D.	N.D.
estrone	535 ± 62	697 ± 82	93 ± 23	107 ± 22	68 ± 5
medroxyprogesterone	N.D.	N.D.	N.D.	N.D.	N.D.
progesterone	<LOQ	196 ± 37	N.D.	N.D.	N.D.
total	2103 ± 123	1101 ± 93	154 ± 28	161 ± 26	76 ± 6

[a] Data shown are mean with standard deviation from six samples for each location. [b] Estimated age of each sample. [c] N.D.: not detected.

followed by estrone, 17β-estradiol, and progesterone (Figure 3). For the piled manure which stemmed from fresh manure stocked in a dry area for two weeks, estrone was detected as the most important hormone with the highest concentration of 697 μg/kg. This finding is consistent with the previous results that indicated that 17α-estradiol may rapidly be transformed to estrone by manure microorganisms. Also, the total dry basis hormone concentration in the piled manure was about half of that in the fresh manure (Table 1). From the point of view of prevention from hormone release to the environment, manure piling may be one simple and economically feasible manure management practice that allows time for the biodegradation of hormones, which may thereby reduce the potential loading to agricultural fields and decrease the environmental risks associated with these EDCs.

Three of the six hormones were detected in settled solid wastes that were collected from the three slotted dams. The dry basis concentration of each hormone in the settled solid manure is significantly less than that in fresh or piled manure (Table 1). This may be due to flushing of large amounts of water over these settled solids, or may be due to degradation as the solids accumulated. This result also implies that the hormone load to the environment from settled manure solids is significantly less compared with piled manure when they are applied at the same agronomic rates.

The levels of steroid hormones in all solid wastes ranged from parts-per-billion (10^{-9}, μg/kg) to parts-per-million (10^{-6}, mg/kg). The concentrations of hormones in waste lagoon water were detected in the range of parts-per-trillion (10^{-12}, ng/L). This indicates that the land application of solid wastes is likely linked with more hormone contamination problems in contrast to the use of the waste lagoon water, because the former contains significantly higher concentrations of hormones. Collectively, increasing the piling time of solid wastes and increasing residence time of wastewater in lagoons may increase the biodegradation time for steroid hormones, which implies that they would be efficient strategies to reduce potential contaminated sources of these hormones.

Environmental Significance. In this study, quantification of steroid hormones in dairy wastes clearly illustrates a spatial distribution of these contaminants on a typical dairy farm in California. In the fresh dairy wastewater and solid manure, 17α-estradiol was detected as the primary steroid hormone. However, the load of major hormone contaminant to environment would be estrone since the estradiol was rapidly biotransformed to the latter in the dairy waste disposal system. Estriol as one of main metabolites of estrone was never detected from any dairy samples, which is consistent with previous observations (*1, 11, 23*). In contrast, estrone may be further transformed to estriol in swine sow and poultry lagoons (*3, 11*). This discrepancy may be attributable to the differences in degradation potential due to the microbial community that is present in the lagoon water from various CAFOs. The detailed transformation mechanism and pathway of estrone in dairy wastes needs to be further investigated. Progesterone, as an important gestagen hormone, was detected in the piled manure and the contribution of this gestagen to the overall hormone load could not be ignored. Medroxyprogesterone is a synthetic gestagen used in human and veterinary medicine and has been detected in dairy lagoon effluents and corral wells (*1*). Although this chemical was not detected in the present study, except in a casual piled waste sample, it indicates that other synthetic veterinary pharmaceuticals, antibiotics, and growth promoters used in animal agriculture could be present in these wastes and potentially threaten the environment near dairies. Also, testosterone, an important androgen steroid hormone which often occurs in many other CAFO wastes, was not detected on the dairy farm (data not shown). It appears that the occurrence and abundance of steroid hormones will vary with animal type, management, and waste disposal system.

Hormones are excreted from livestock species as either free hormones or as sulfate or glucuronide conjugates. The present study focused on the free steroid hormones. A recent study showed that estrogen conjugates contribute significantly to the overall estrogen load from different types of CAFO lagoons (*11*). It was estimated that estrogen conjugates account for at least a third of the total estrogen equivalents in most cases, or even more because of incomplete enzymatic hydrolysis. Although most conjugated forms are biologically inactive, they may act as precursor hormone reservoirs that may be readily deconjugated by bacteria to produce corresponding active free hormones. The effect of hormone conjugates on endocrine-disrupting activities still needs to be considered and further study should be conducted to investigate the transformation between free and conjugate steroid hormones in dairy wastes.

Currently, the Environmental Protection Agency (EPA) requires that CAFO waste application to agricultural fields comply with a Comprehensive Nutrient Management Plan (CNMP). This approach assumes that a well-designed CNMP will ensure that all contaminants are retained in the root zone, so that groundwater and surface water are inherently protected. To minimize the potential contamination of active hormones, it may be necessary to reduce the load of total steroid hormones from CAFOs waste prior to land application. This study indicates that the use of sequencing lagoons and increasing manure-piling time may offer feasible, efficient, and inexpensive practices to eliminate hormone contaminants.

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Supporting Information Available

A typical dairy waste disposal system and its diagram, method performance, tables showing coefficients of variation for the yields of HFB derivatives and method validation parameters, and figures showing selected ion chromatogram of steroid hormones in dairy manure wastewater, electron impact (EI) mass spectra of HFB hormone derivatives, and hormone concentrations in dairy wastewater and waste lagoon water. This material is available free of charge via the Internet at http://pubs.acs.org.

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