Environ. Sci. Technol. **2006**, *40,* 7896−7902

Occurrence of Estrogenic Compounds in and Removal by a Swine Farm Waste Treatment Plant

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The total estrogenic activity of the wastewater from a swine farm in Japan was quantitatively characterized, and the compounds responsible for the estrogenic activity were identified and quantified. The wastewater treatment process consisted of a series of an up-flow anaerobic sludge blanket (UASB) and a trickling filter. Samples were collected at each treatment step, and the total estrogenic activity was determined by use of an in vitro gene expression assay (MVLN; MCF-7 human breast cancer cell stably transfected with the pVit-tk-LUC receptor plasmid). Individual estrogenic compounds were identified and quantified using liquid chromatography-mass spectrometry (LC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). To further identify the compounds contributing to the estrogenic activity in the wastewater, the sample extracts were fractionated into 12 fractions (fractions 1−12) by HPLC. The rate of removal of estrogenic activity between the effluent and the influent was greater than 97%. The trickling filter removed the majority of the estrogenic activity. The removal rates of specific estrogenic compounds ranged from 44 to 99%. Estrogenic activity was detected mainly in the fractions containing estrone (E1), 17β-estradiol (βE2), 17α-estradiol (αE2), estriol (E3), bisphenol A (BPA), and equol (EQO). The ratios of βE2-EQ_C (βE2 equivalents derived from chemical analysis) to βE2-EQ_B (βE2 equivalent derived from bioassay) in the 12 fractions collectively were contributed by E1 (17−30%), βE2 (23−30%), αE2 (<1%), E3 (1−2%), BPA (<1%), and EQO (2−3%) in the influent and E1 (16−37%), βE2 (<1−7%), αE2 (<1%), E3 (<1−3%), BPA (<1%), and EQO (<1%) in the effluent. The compounds responsible for most of the estrogenic activity measured in the bioassay were natural estrogens such as E1 and βE2.

Introduction

The occurrence of estrogenic compounds in aquatic environments and the effects on normal endocrine function in aquatic organisms have been subjects of current concern (*1*−*3*). Steroidal estrogens such as 17β-estradiol (βE2) and estrone (E1) are mainly eliminated from vertebrates and are released into rivers and estuaries via wastewater treatment plants (*4*−*8*) and also via runoff from agricultural land through livestock excreta (*9*−*13*). Estrogenic compounds from agricultural activities are particularly important because livestock excreta are a major source of estrogens to the aquatic environment (*9*−*11*). In the United States, the total daily emission of βE2 and E1 from dairy and swine have been reported to range from 10 to 30 kg and from 20 to 80 kg, respectively. These values are greater than the mass flow of estrogen from municipal sewage treatment plants in the United States (*10*). Annual excretion of estrogens by farm animals, including cattle, pigs, sheep, and chickens, has been estimated to be 39 tons in the European Union and 41 tons in the United States (*11*). Concentrations of βE2 in farm ponds receiving cattle runoffs ranged from 0.05 to 7.4 ng/L; these concentrations can elicit effects on the normal reproduction of turtles in the ponds (*12*). Indeed, 3−4-fold greater concentrations of estrogens were observed in wastewater originating from agricultural activities than from municipal wastes (*13*). Concentrations of E1 in well water near a swine farm have been reported to be 4.5 ng E1/L (*14*). This suggests that agricultural activities can not only affect surface waters but also groundwater (*14*). Despite the role of agricultural operations in contamination of surface and groundwater, few studies have focused on the contamination by steroidal hormones released from various farm-related activities. Furthermore, the extent of the need for and the impact of the treatment of wastewater released from farming activities to remove steroidal compounds are not known.

In November 2004, the Ministry of Agriculture, Forestry and Fisheries of Japan revised the law for the recycling of farm wastes by imposing stringent rules designed to minimize the environmental impact of wastes from livestock farms; wastewater treatment facilities were built to treat farm wastes, with the aim of reducing biological oxygen demand (BOD), suspended solids (SS), odor, nitrogen, and phosphorus in effluent waters. However, no attention was paid to the release of and treatment for steroidal hormones and other toxic substances.

The objectives of the present study were to (i) measure the estrogenic activity in swine farm wastewater using an in vitro gene expression MVLN cell (MCF-7 human breast cancer cell stably transfected with a pVit-tk-LUC receptor plasmid) bioassay; (ii) identify and quantify various potentially estrogenic compounds using liquid chromatography−mass spectrometry (LC/MS) or liquid chromatography-tandem mass spectrometry (LC/MS/MS) at each treatment stages at

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10.1021/es0609598 CCC: $33.50
Published on Web 11/07/2006

a pilot-scale swinery waste treatment plant; (iii) assess the rates of removal of estrogenic activity and estrogenic compounds in the wastewater treatment process involving an up-flow anaerobic sludge blanket (UASB) and a trickling filter; and (iv) quantitatively characterize the compounds contributing to estrogenic activity, with bioassay, instrumental analysis, and fractionation.

Materials and Methods

Sample Collection. Wastewater samples were collected on July 9, 2003 and October 6, 2003 from several stages in a pilot-scale swine farm wastewater treatment plant at the National Institute of Livestock and Grassland Sciences, Tsukuba, Japan (*15*) (Supporting Information 1). Grab sampling was conducted at three points: (i) inlet, (ii) UASB outlet water, and (iii) trickling filter effluent water. The samples were transferred to glass bottles and kept in a cooler at 4 °C and then brought to the laboratory. Extraction of the samples was performed on the same day, to prevent biodegradation of target compounds.

Extraction. Samples were extracted by a concentration system with flow control (Nippon Waters Corporation, Tokyo, Japan), equipped with a 1 μm (pore size) glass-fiber filter (preheated at 400 °C for 4 h prior to use). A solid-phase extraction (SPE) cartridge filled with hydrophilic copolymer including *N*-vinylacetamide (EDS-1; 500 mg; Showadenko Corporation, Tokyo, Japan) precleaned with 10 mL of methanol and 10 mL of ultrapure water (nanopure water; Millipore Corporation, Billerica, MA) was prepared. After the pH of the samples was adjusted to 3 with acetic acid, the water samples were passed through this system at a flow rate of 10 mL/min. The sample volume used for extraction was 75−100 mL for inlet water and UASB outlet water and 330−400 mL for trickling filter effluent water. Blanks (1000 mL) were prepared by the passage of nanopure water through the extraction procedure. After passage of the samples, residual water within the cartridges was removed by centrifugation at 1000 rpm for 10 min. Cartridges were then eluted with 10 mL of 5 mM trimethylamine in methanol, into glass centrifuge tubes. The eluants were concentrated under a gentle stream of nitrogen at 30 °C. After drying, 500 μL of methanol was added to the tubes, and 200 μL of the extracts was prepared for MVLN bioassay as a crude sample extract. A 200 μL aliquant of extract was used for identification of the estrogenic activity in the samples. Fractionation of the extracts (200 μL) was performed only after the presence of estrogenic activity was confirmed by the bioassay.

Fractionation. Crude extracts were fractionated into 12 fractions (fractions 1−12) by gel-permeation chromatography (GF-310Q) (300 mm × 7.6 mm φ, Showadenko Corporation, Tokyo, Japan) (Supporting Information 2). A volume of 200 μL of crude extract was injected into the HPLC system. Each fraction was collected using the electronic fraction collector at 4 min intervals. Fractionated samples were concentrated under a gentle stream of nitrogen at 30 °C. After drying, 200 μL of methanol was added for the bioassay. After conducting the bioassay, 170 μL of the extract of each fraction was transferred into a vial using a micropipet and concentrated under a gentle stream of nitrogen at 30 °C to dryness. A volume of 170 μL containing the mixture of 17β-estradiol-d_4, estrone-d_2, and estriol-d_2 was added as internal standards. This resulted in a final concentration of each of the three deuterated internal standards of 50 μg/L.

Target Compounds. Target compounds were selected based on their potential hormonal activity in the aquatic environment: E1, αE2, βE2, E3, and EE2 as hormonal estrogens; GE and EQO as phytoestrogens; and NP, OP, and BPA as synthetic estrogens. EE2 is not used in swine farms. 17β-estradiol (βE2, 98%), 17α-estradiol (αE2, 98%), estrone (E1, 99%), estriol (E3, 99%), genistein (GE, 99%), and ethynylestradiol (EE2, 98%) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). 4-Nonylphenol (NP, 99%), 4-tert-octylphenol (OP, 98%), and bisphenol A (BPA, 99%) were obtained from Wako Chemical Industries, Ltd. (Osaka, Japan). Equol (EQO, 99%) was obtained from Fluka Chemie (Buchs, Switzerland). The deuterated internal standards, E1-d_2 (E1-2, 4-d_2), βE2-d_4 (βE2-2, 4, 16, 16-d_4), and E3-d_2 (E3-2, 4-d_2) were obtained from CDN Isotopes (Pointe Claire, Quebec, Canada). All solvents used in the experiments were pesticide grade.

Instrumental Analysis. Target compounds in water samples were identified, and their concentrations were determined by use of either LC/MS/MS (Quattro Ultima; Micromass, Manchester, UK) or LC/MS (MSD1100; Agilent, Palo Alto, CA). The HPLC systems employed for LC/MS/MS and LC/MS were gradient systems consisting of Alliance2695 (Waters, Milford, MA) and HP1100 series (Agilent), respectively (Supporting Information 3). Recoveries of target compounds are given (Supporting Information 4).

Bioassay. Estrogenic activities were determined by use of the MVLN trans-activation assay. The method has been described by Snyder et al. (*16*), and additional study-specific information is provided (Supporting Information 5). MVLN cells are derived from the human breast carcinoma MCF-7 cell line and are stably transfected with a luciferase reporter gene under the control of estrogen-responsive elements (EREs) of the *Xenopus vitellogenin* A2 gene, for the detection of estrogenic receptor (ER)-meditated activity (*17*).

Bioassay Data Analysis. Estrogenic activity, expressed as mean relative luminescence unit (RLU) of the three replicates, was converted into the ratio of the mean maximum response observed for the βE2 standard curve generated daily. The maximum response ratio of βE2 was set as 1. The estrogenic activity in samples derived from the bioassay analysis was expressed as the equivalent quantity of βE2 (βE2-EQ_B and ng of βE2-eq/L), which was estimated from a logistic model (*18*, *19*) (Supporting Information 6).

Determination of βE2 Equivalents from Chemical Analysis (βE2-EQ_C). The βE2 equivalent quantity from chemical analysis, expressed as βE2-EQ_C (ng of βE2-eq/L), was calculated as the sum or the products of the measured concentration of each target estrogenic compound and its relative potency (REP). It was assumed that the estrogenic responses measured in the bioassay were additive over different estrogenic compounds present in the samples. On the basis of the results, the contribution of each target compound to the total estrogenic activity in the samples was determined. The REP values of each estrogenic chemical were determined empirically in this study (Supporting Information 4).

Results and Discussion

Estrogenic Activity of Crude Extracts. Crude extracts of wastewaters elicited luciferace activity as measured by light emission by MVLN cells (Supporting Information 7), and the βE2-EQ_B of crude samples (βE2-$EQ_{B:crude\ extract}$) are reported (Figure 1 and Supporting Information 7).

Estrogenic Compounds. Concentrations of target compounds are reported for each fraction that was associated with each type of estrogenic compound (Table 1). In the raw swine wastewater, concentrations of βE2 were 1000 ng/L (July) and 1500 ng/L (October). Concentrations of E1 (5200 and 5400 ng/L) in the raw swine wastewater in our study were 4−5-fold greater than concentrations of βE2. Measured concentrations of E3 were 2200 and 3000 ng/L for the two time periods, which were almost twice as great as those of βE2 in raw swine wastewater. An earlier study showed that E1 and E3 concentrations were greater than that of βE2 in samples of swine farm waste lagoons (*14*). E1 has previously been reported to be the major estrogenic compound in swine

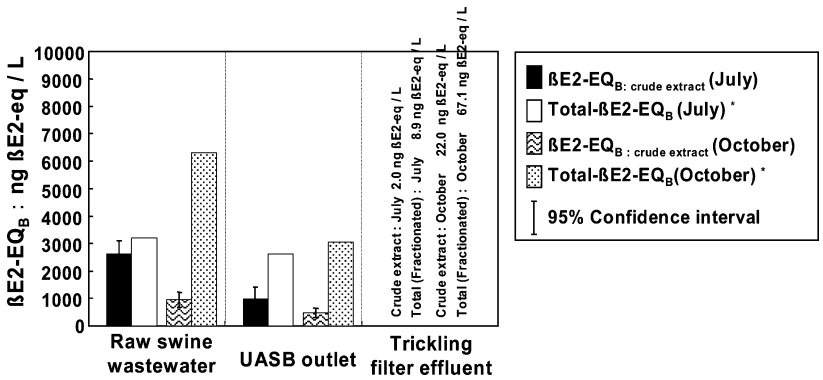

FIGURE 1. Bioassay-derived estradiol equivalents in crude swine farm wastewater (βE2-EQ$_{B:crude\ extracts}$) and fractionated extract (total-βE2-EQ$_B$) in each step of the treatment process. *βE2-EQ$_B$ of fraction was the sum of βE2-EQ$_B$ obtained from each fraction.

farm wastes (*10*). In our study, concentrations of αE2, which were 650 ng/L in July and 680 ng/L in October, were less than those of βE2. αE2, the main metabolite of βE2 in cattle excrement (*9*, *20*), has been found to be the predominant estrogenic compound in solid dairy wastes and in dairy waste holding ponds (*10*). To confirm the existence of αE2 in raw cattle waste, we sampled a wastewater inlet from a cattle farm, also at the National Institute of Livestock and Grassland Sciences, Japan. The concentration of αE2 in the cattle wastewater inlet was 560 ng/L (July) and 2000 ng/L (October), which was significantly greater than the concentrations of βE2 (12 ng/L in July and 60 ng/L in October). The difference in concentrations of βE2 and αE2 between swine and cattle wastes has been reported to be due to differing metabolisms by the two species (*9*). The concentration of EQO, a phytoestrogen, was 0.94 mg/L in July and 1.1 mg/L in October in raw swine wastewater. Swine waste has previously been determined to have an EQO concentration of 6.9−16.6 mg/L (*21*). Metabolites of phyoestrogens such as daizein, GE, and EQO have been found in the urine of several animals, including horses, goats, sheep, and hens (*22*). However, the concentration of GE in the raw swine wastewater in our study was found to be less than the limit of detection (Table 1). The concentrations of NP, OP, and BPA were 1200−2300, 96−120, and 1100−1200 ng/L for July and October, respectively. NP and OP detected in the raw swine wastewater were likely derived from detergent used in the swine house. Although the source of BPA was not clear, feed items or other equipment used in swine farm could have been a possible source. The concentration of EE2 in the raw swine wastewater was found to be less than the limit of detection (Table 1).

In the trickling filter effluent, natural hormones, including βE2 and EQO, were detected in samples from July, while E1, βE2, αE2, E3, and EQO were detected in samples from October. The concentration of E1 was 17-fold greater in samples collected in October than those collected in July. Concentrations of other compounds such as βE2 (4.5 ng/L), αE2 (24 ng/L), E3 (72 ng/L), and EQO (41 ng/L) in samples collected in October were greater than the concentrations found in July. These results coincided with the values for BOD and SS in the trickling filter effluent (Supporting Information 1). The difference in concentrations of estrogens in the trickling filter effluent observed in July and October may be related to the inlet concentrations, temperature, and treatment efficiency. No differences in concentrations of synthetic estrogens, NP, OP, and BPA were observed between the samples collected in July or October. Concentrations of NP, BPA, and OP observed in our study were similar to those previously reported for sewage treatment plant effluents in Japan (*23*).

Rates of removal of estrogenic compounds from the influent (raw swine wastewater) ranged from 44 to >99% (calculated from the data listed in Table 1). In particular, natural estrogens such as E1, βE2, αE2, E3, and EQO were removed efficiently (96 to >99%), while synthetic estrogenic compounds such as NP, OP, and BPA were removed less efficiently (46−85%) in the treatment process. Between the stage of the raw swine wastewater and the stage of the UASB outlet, the EQO concentration decreased by 94%, while the other compounds (except for BPA) were reduced by 2−78%. In particular, αE2 was reduced by less than 10% (9.6% in July and 2.2% in October). E3 and BPA concentrations decreased by only 9% in October. The poor removal of estrogenic compounds in the UASB outlet indicates that target compounds are less efficiently degraded under anaerobic conditions than under aerobic conditions. It has been reported that βE2, EE2, BPA, NP, and OP are less degradable under anaerobic conditions (*24*). The concentration of BPA increased by approximately 1.7 times in the UASB effluent relative to that in raw wastewater in July and October. This result might be due to a variance of sampling, as we used grab sampling. Further investigation of the degradation of BPA in this plant is needed. In general, natural estrogens, E1, βE2, αE2, E3, and EQO, were more degradable than synthetic compounds such as NP, OP, and BPA in this treatment process.

Estrogenic Activity. Concentrations of βE2-EQ$_B$ in each fraction were summed to give total βE2 equivalent and to permit calculation of the rates of removal by each treatment process (Figure 1). The rate of removal of total βE2-EQ$_B$ by the UASB outlet relative to the rate in the raw swine wastewater ranged from 19 to 50%. This indicates that estrogenic activity was not efficiently removed under anaerobic conditions. This result, based on the total E2 equivalent, is in agreement with the result of the instrumental analysis of estrogenic compounds. The reduction in total βE2-EQ$_B$ was significant in the trickling filter process, and the rate of removal of estrogenic activity relative to the raw swine wastewater was over 97%. In a previous study, aerobic treatment of biosolids obtained from a sewage plant caused a significant reduction in estrogenic activity (*25*). Thus, the results of this study support the previous finding of significant reduction in estrogenic activity under aerobic conditions.

In October, the concentration of E1 in trickling filter effluent was 120 ng/L. This is almost 3-fold greater than the concentration at which vitellogenin (VTG) is induced in adult rainbow trout (>44 ng/L) (*3*). The rate of removal of E1 was 64.7% in our study. This rate is less than those that have been previously reported to be less than the rates of removal of βE2 (81.7%) and EE2 (85.2%) in an activated sludge treatment process (*26*). The explanation proposed to account for that pattern of removal was oxidative transformation of βE2 and E1-sulfate to E1 in the activated sludge treatment process (*8*). The total βE2-EQ$_B$ of fractionated extracts was 1.3−6.7-fold greater than that of βE2-EQ$_B$ of the crude extract,

TABLE 1. Concentrations (ng/L) of Target Estrogenic Compounds at Each Step of the Treatment Process in a Swine Farm Wastewater Treatment Plant[a]

July 2003

		concentration (ng/L)								
		fraction number								
sampling site	estrogenic substance	F5	F6	F7	F8	F9	F10	F11	F12	total
raw swine wastewater	estrone (E1)				3.8×10^3	1.4×10^3	5.0×10^1			5.2×10^3
	17β-estradiol (βE2)			1.0×10^3						1.0×10^3
	17α-estradiol (αE2)			6.5×10^2						6.5×10^2
	estriol (E3)			2.2×10^3						2.2×10^3
	ethnylestradiol (EE2)			<6.3						<6.3
	4-*t*-octylphenol (OP)		1.2×10^2							1.2×10^2
	4-nonylphenol (NP)		2.3×10^3							2.3×10^3
	bisphenol A (BPA)			1.0×10^3	8.4×10^1					1.1×10^3
	genistein (GE)	<0.4								<0.4
	equol (EQO)						9.1×10^5	3.4×10^4	2.9×10^3	9.4×10^5
UASB outlet	estrone (E1)				2.8×10^3	9.9×10^2	3.2×10^1			3.8×10^3
	17β-estradiol (βE2)			4.7×10^2						4.7×10^2
	17α-estradiol (αE2)			5.9×10^2						5.9×10^2
	estriol (E3)			1.5×10^3						1.5×10^3
	ethnylestradiol (EE2)			<6.3						<6.3
	4-*t*-octylphenol (OP)		3.9×10^1							3.9×10^1
	4-nonylphenol (NP)		9.7×10^2							9.7×10^2
	bisphenol A (BPA)			1.6×10^3	2.8×10^2					1.9×10^3
	genistein (GE)	<0.4								<0.4
	equol (EQO)						5.2×10^4	2.3×10^3	2.2×10^2	5.5×10^4
trickling filter effluent	estrone (E1)				6.1	0.8				6.9
	17β-estradiol (βE2)			<0.3						<0.3
	17α-estradiol (αE2)			<0.3						<0.3
	estriol (E3)			<0.2						<0.2
	ethnylestradiol (EE2)			<1.4						<1.4
	4-*t*-octylphenol (OP)		1.9×10^1							1.9×10^1
	4-nonylphenol (NP)		4.5×10^2							4.5×10^2
	bisphenol A (BPA)			4.2×10^2	6.5×10^1					4.9×10^2
	genistein (GE)	<0.1								<0.1
	equol (EQO)						1.6×10^2	1.1×10^1	<0.1	1.7×10^2

October 2003

		concentration (ng/L)								
		fraction number								
sampling site	estrogenic substance	F5	F6	F7	F8	F9	F10	F11	F12	total
raw swine wastewater	estrone (E1)				3.6×10^3	1.8×10^3	3.9×10^1			5.4×10^3
	17β-estradiol (βE2)			1.5×10^3						1.5×10^3
	17α-estradiol (αE2)			6.8×10^2						6.8×10^2
	estriol (E3)			3.0×10^3						3.0×10^3
	ethnylestradiol (EE2)			<9.5						<9.5
	4-*t*-octylphenol (OP)		9.6×10^1							9.6×10^1
	4-nonylphenol (NP)		1.2×10^3							1.2×10^3
	bisphenol A (BPA)			1.0×10^3	2.0×10^2					1.2×10^3
	genistein (GE)	<0.6								<0.6
	equol (EQO)						1.0×10^6	7.0×10^4	6.0×10^3	1.1×10^6
UASB outlet	estrone (E1)				2.4×10^3	1.1×10^3	2.4×10^1			3.6×10^3
	17β-estradiol (βE2)			4.1×10^2						4.1×10^2
	17α-estradiol (αE2)			6.6×10^2						6.6×10^2
	estriol (E3)			2.7×10^3						2.7×10^3
	ethnylestradiol (EE2)			<9.5						<9.5
	4-*t*-octylphenol (OP)		8.7×10^1							8.7×10^1
	4-nonylphenol (NP)		5.6×10^2							5.6×10^2
	bisphenol A (BPA)			1.4×10^3	6.1×10^2					2.0×10^3
	genistein (GE)	<0.6								<0.6
	equol (EQO)						3.2×10^4	3.5×10^3	2.0×10^2	3.6×10^4
trickling filter effluent	estrone (E1)				9.1×10^1	3.3×10^1	0.4			1.2×10^2
	17β-estradiol (βE2)			4.5						4.5
	17α-estradiol (αE2)			2.4×10^1						2.4×10^1
	estriol (E3)			7.2×10^1						7.2×10^1
	ethnylestradiol (EE2)			<6.9						<6.9
	4-*t*-octylphenol (OP)		1.8×10^1							1.8×10^1
	4-nonylphenol (NP)		3.1×10^2							3.1×10^2
	Bisphenol A (BPA)			6.0×10^2	4.7×10^1					6.4×10^2
	genistein (GE)	<0.1								<0.1
	equol (EQO)						3.8×10^2	2.7×10^1	1.6	4.1×10^2

[a] Samples collected in July 2003 and October 2003.

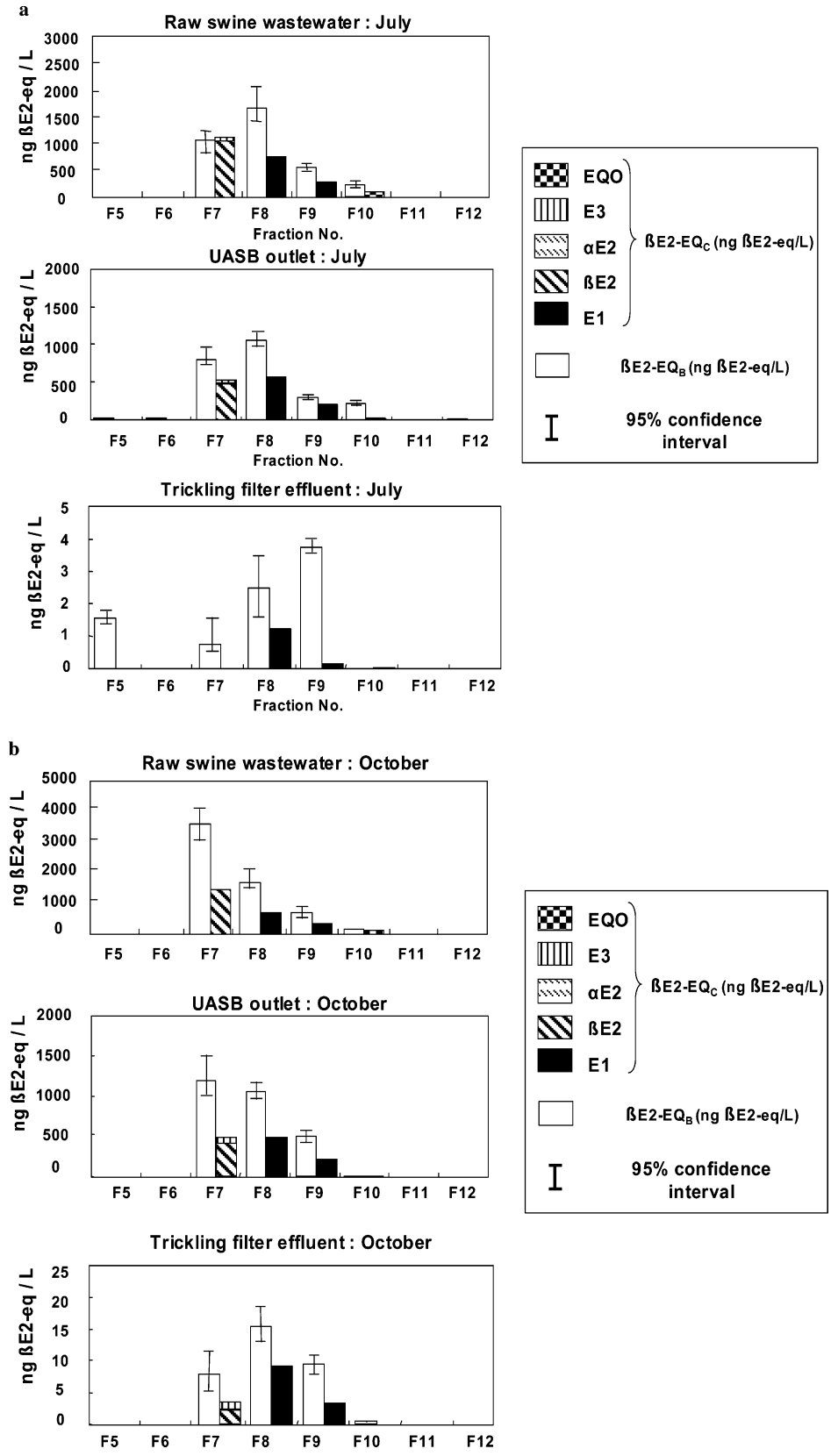

FIGURE 2. (a) Comparison of 17β-estradiol equivalents (ng of βE2-eq/L) between chemical analysis (βE2-EQ$_C$) and bioassay analysis (βE2-EQ$_B$) in fractionated samples for each step of the treatment process, collected in July 2003. (b) Comparison of 17β-estradiol equivalents (ng of βE2-eq/L) between chemical analysis (βE2-EQ$_C$) and bioassay analysis (βE2-EQ$_B$) in fractionated samples for each step of the treatment process, collected in October 2003.

indicating that the fractionation was useful in reducing the matrix and cytotoxic substances (Figure 1).

Mass Balance Analysis of Estrogenic Compound to Estrogenic Activity. To assess the contribution of known estrogenic compounds to total estrogenic activity, concentrations of βE2-EQ$_B$ and βE2-EQ$_C$ were compared (Figure 3). The concentration of βE2-EQ$_C$ in each fraction was calculated by multiplication of the measured concentration of each

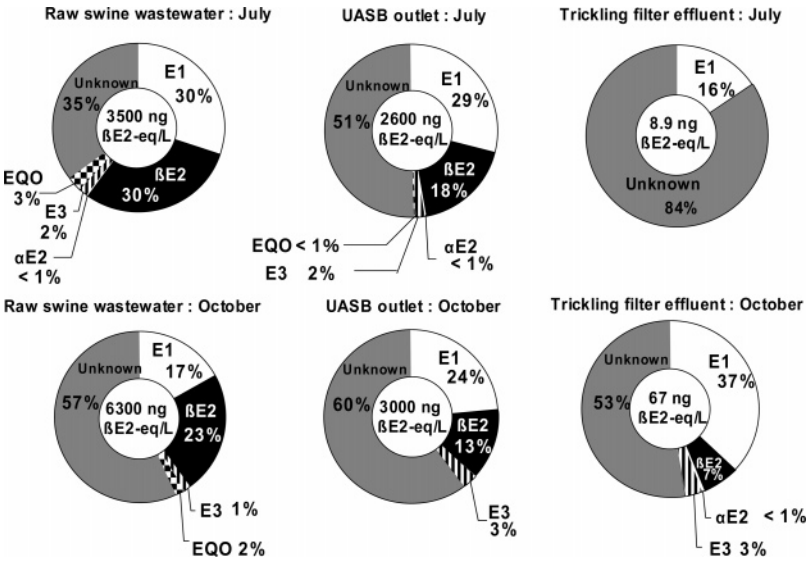

FIGURE 3. Contribution of each target estrogenic compound to the total estradiol equivalents (βE2-EQ$_B$). Total βE2-EQ$_B$ was obtained as the summation of all fractions of the wastewater extract. EE2 and GE were not detected. The 17β-estradiol equivalents of NP and OP, derived from chemical analysis, were relatively small.

compound with the compound's REP, and the βE2-EQ$_C$ values were summed to give total estradiol equivalents of the fraction. REPs for αE2, E3, and BPA measured by MVLN cells were 2.3×10^{-3}, 2.6×10^{-2}, and 3.0×10^{-7}, respectively.

In the raw swine wastewater, estrogenic activity was found in fractions 7−10 (F7−F10). LC/MS and LC/MS/MS analyses revealed the presence of βE2, αE2, E3, and BPA in F7 (Table 1). The concentration of βE2-EQ$_C$ was calculated by multiplication of the REP and measured concentration of each compound. In F7, βE2 contributed the majority of the βE2-EQ$_B$ measured in that fraction (Figure 2). In particular, in samples of F7 collected in July, βE2 accounted for almost all of the βE2-EQ$_B$. In F8, βE2-EQ$_C$ of E1 was responsible for half the concentration of βE2-EQ$_B$. In F10, in October, βE2-EQ$_B$ could be explained largely on the basis of EQO, which accounted for about 80% of βE2-EQ$_C$. Although E1 was measured in F10 in each sample, only a portion of the βE2-EQ$_B$ values, 5% in July and 20% in October, was accounted for by βE2-EQ$_C$ of E1.

The observed trend in total estrogenic activity in the UASB outlet was similar to that of the raw swine wastewater, if βE2-EQ$_B$ and βE2-EQ$_C$ from F7−F10 are compared. Particularly, in F5, F6, and F12 for July sampling, the βE2-EQ$_B$ values were 31, 27, and 25 ng of βE2-eq/L, respectively. GE eluted in F5 was less than the detection limit (0.4 ng/L). In F6, βE2-EQ$_c$ of NP and OP concentrations ranged from 2.9×10^{-3} to 1.5×10^{-4} ng of βE2-eq/L, concentrations that would be less than the sensitivity of the MVLN cell assay (<0.7 ng of βE2-eq/L) and consistent with the results of βE2-EQ$_B$. In F12, EQO was detected. The βE2-EQ$_C$ of EQO was 2.9×10^{-3} ng of βE2-eq/L, and this value does not account for the βE2-EQ$_B$ value. The results suggest that unknown estrogenic compounds are responsible for the activity in this fraction.

In the trickling filter effluent, estrogenic activities were observed in F5, F7, F8, and F9. In F8, βE2-EQ$_c$ of E1 was responsible for approximately 50% of the βE2-EQ$_B$ value. The βE2-EQ$_B$ values for F5, F7, and F9 were not accounted for by target compounds analyzed in this study. In samples collected in October, the βE2-EQ$_B$ values in F7, F8, F9, and F10 were largely attributable to βE2-EQ$_C$ of E1 and βE2. However, the target compounds in this study could not explain all of the βE2-EQ$_B$ in each fraction.

Concentrations of βE2-EQ$_B$ for each compound were summed to calculate the contribution by the target compounds to estrogenic activity, expressed as total βE2-EQ$_B$ (Figure 3). The ratios of βE2-EQ$_C$ to βE2-EQ$_B$ in the fractions were E1, 17−30%; βE2, 23−30%; αE2, <1%; E3, 1−2%; BPA, <1%; EQO, 2−3% in the raw wastewater and E1, 16−37%; βE2, <1−7%; αE2, <1%; E3, <1−3%; BPA, <1%; and EQO, <1% in the trickling filter effluent. These results indicate that the principal compounds contributing to the estrogenic activity were natural estrogens such as E1 and βE2. The existence of unknown estrogenic compounds in the raw swine wastewater was also observed. As described before, estrogenic compounds in F7−F10, including E1 and βE2, could not account for all of the βE2-EQ$_B$. Therefore, unknown estrogenic compounds exist in these fractions. E1 and βE2 have previously been reported to contribute only a minor proportion, whereas EQO contributed significantly to the estrogenic activity in hog manure (*21*). However, in our study, EQO contributed little to the estrogenic activity. The REP of yeast bioassay for EQO was previously reported to be 1×10^{-3} (*21*); in contrast, the REP that we calculated for EQO in the MVLN assay was approximately 35-fold less, 2.8×10^{-5} (Supporting Information 4).

Several other compounds that are known to elicit estrogenic activity were not measured in this study. Testosterone and dihydrotestosterone are androgens and have been shown to elicit estrogenic activity with a relative potency of 1×10^{-5} in MVLN cells (*27*). Sow manure has been shown to elicit androgenic activity (*25*). Steroids, such as testosterone and androstenedione, have been detected at concentrations of a few or tens of nanograms per liter in municipal wastewater effluent (*28*). Phytoestrogens such as daizein, formononetin, biochanin, GE, flavone, glycitein, trihydroxyisoflavone, and coumestrol, which have been detected in sewage treatment effluents in the range of several tens to hundreds of nanograms per liter (*29*), might contribute to the undetermined estrogenic activity.

The results of the present study suggest that wastewater from swine farms can be a source of estrogenic compounds to the environment. Estrogenic activity and estrogenic compounds can be removed by aerobic treatment processes involving trickling filters. Whereas natural estrogens are the major contributors to the estrogenic activity of swine farm wastes, such activities can be efficiently be removed by appropriate treatment. Synthetic estrogenic compounds such as NP, OP, and BPA are removed relatively less efficiently. Mass balance analysis of estrogenic activity and estrogenic compounds, using in vitro gene expression analysis and

instrumental analysis, is an effective approach to understand critical contaminants of concern in wastewaters and also to identify the existence of unknown compounds. The results of this study suggest the need to monitoring the sources and treatment of toxic substances released from farming activities.

Acknowledgments

Financial support by a "Grant-in-Aid for the Creation of Innovations through Business-Academic-Public Sector Cooperation" (12323) and the 21st Century COE Program "Bio-Eco Environmental Risk Management", Ministry of Education, Culture, Sports, Science and Technology are gratefully acknowledged.

Supporting Information Available

Supplemental table and figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review April 20, 2006. Revised manuscript received August 27, 2006. Accepted September 25, 2006.

ES0609598