Studies reveal that surface waters worldwide are contaminated with hormonally active agents, many released from sewage treatment plants. Another potential source of aquatic hormonal contamination is livestock feedlot effluent. In this study, we assessed whether feedlot effluent contaminates watercourses by measuring $a)$ total androgenic [methyltrienolone (R1881) equivalents] and estrogenic (17β-estradiol equivalents) activity using the A-SCREEN and E-SCREEN bioassays and $b)$ concentrations of anabolic agents via gas chromatography–mass spectroscopy and enzyme-based immunoassays. 

Water samples were collected over 3 years from up to six sites [all confluent with the Elkhorn River, Nebraska, USA: a feedlot retention pond (site 1), a site downstream from site 1 (site 2), a stream with intermediate livestock impact (site 3), and three sites with no observable livestock impact (sites 4–6)] and two sources of tap water. In 1999, samples from site 1 contained 9.6 pM R1881 equivalents and 1.7 pM 17β-estradiol equivalents. Site 2 samples had estrogen levels similar to those in site 1 samples but lower androgen levels (3.8 pM R1881 equivalents). Androgen levels in site 3 samples were similar to those in site 2 samples, whereas estrogen levels decreased to 0.7 pM 17β-estradiol equivalents. At site 6, androgen levels were approximately half those found at site 3, and estrogen levels were comparable with those at site 3. Sampling in later years was limited to fewer sites because of drought and lack of permission to access one site. Instrumental analysis revealed estrone but no significant levels of resorcylic acid lactones or trenbolone metabolites. Tap water was devoid of hormonal activity. We conclude that feedlot effluents contain sufficient levels of hormonally active agents to warrant further investigation of possible effects on aquatic ecosystem health.

Key words: agricultural runoff, anabolic steroid hormones, aquatic ecosystem health, A-SCREEN, concentrated animal feeding operations (CAFOs), environmental androgens, environmental estrogens, E-SCREEN, personal care products, pharmaceuticals. Environ Health Perspect 112:346–352 (2004). doi:10.1289/ehp.6590 available via http://dx.doi.org/ [Online 1 December 2003]
natural hormones, anabolic steroids, and metabolites into the environment. The downstream hormonal contamination of such operations would potentially alter the reproductive and endocrine systems of exposed organisms.

**Materials and Methods**

**Supplies.** We purchased melengestrol from Biomol Research Laboratories (Plymouth Meeting, PA, USA); 17β-E₂ from Calbiochem (San Diego, CA, USA); and methyltrienolone (R1881) from New England Nuclear (Perkin-Elmer Life Sciences, Boston, MA, USA). Francois André (LABERCA-National Reference Laboratory, Nantes, France) provided trenbolone-17α (Tb-17α), Tb-17β, and trenbolone (TbO). All other hormones were obtained from Sigma Chemical Company (St. Louis, MO, USA). Immunoaffinity chromatography columns for Tb and 19-nortestosterone (catalog no. TB 2186) were purchased from Randox Laboratories (San Diego, CA, USA); and methyltrienolone supplies.

**Research sites.** This study complements one by Orlando et al. (2004) on the endocrine-disrupting effects of cattle feedlot effluent on the fathead minnow, *Pimephales promelas*. Water was collected over a 3-year period from six sites confluent with the Elkhorn River in Nebraska (Figure 1). In June 1999, water samples were obtained from a cattle effluent holding pond directly below a feedlot (retention pond: site 1), a drainage canal 0.5 km downstream that channels water from the feedlot into the Elkhorn River (contaminated site: site 2), a stream draining fields with dispersed cattle and agricultural activity (intermediate exposure site: site 3), and three tributaries of the Elkhorn River with no apparent feedlot activity in the surrounding area (sites 4–6). Only one of these (reference site: site 6) yielded the proper sample size of fathead minnows required by Orlando et al. (2004) for their study. This site was located within the Oak Valley State Wildlife Management Area, approximately 80 km from the feedlot. In addition, tap water samples were acquired from the hose bibs of a recreation center (T1) and a recreational vehicle station center (T2) at a riverside park near site 3. Sampling in September 2000 and July 2001 was limited to sites 1, 3, and 6. Site 2 was not sampled after June 1999 because it was on private land and permission for additional samplings was not obtained. Site 3 was visited in both sampling periods, but drought prevented collection of water during September 2000.

**Test sample preparation and extraction.** Water from the retention pond and streams was collected by immersion of amber glass bottles: September 1999, 4-L bottles precleaned by U.S. Environmental Protection Agency (U.S. EPA) standards (Fisher Scientific, Pittsburgh, PA, USA); September 2000 and July 2001, precleaned 2.5-L bottles (catalog no. 2500-0250; Environmental Sampling Supply, Oakland, CA, USA). Tap water was collected from the hose bibs into the same bottles. We took from 8 L (1999) to 10 L (2000, 2001) from each site and added 0.02% sodium azide to avoid microbial degradation. Southwest Research Institute (SwRI; San Antonio, TX, USA) received the bottles and kept them at 4°C until extraction. SwRI prepared laboratory water blanks from distilled water samples.

We assessed the recovery of compounds likely to be in the field samples by spiking 2 L distilled water samples. Each analyte was spiked to 0.5 µg/L, except for Tb, which was spiked to 2.5 µg/L. We tried three extraction methods: dichloromethane (DCM), 6% ethyl ether–hexane, and 50% ethyl ether–hexane. Because DCM gave the best results, we used this for the field water samples.

Each sample was split into four 1-L fractions in 2-L glass separatory funnels. All glassware was rinsed with 0.6% HCl in DCM and allowed to dry. Each 1-L fraction was extracted three times with 60-mL portions of DCM by shaking 2 min and waiting for at least 10 min before draining the DCM into a 500-mL glass bottle. The DCM extracts of the four fractions were then combined into the same glass bottle. The DCM extracts were concentrated to 2 mL using nitrogen on an N-EVAP evaporator (Organamation Associates, Berlin, MA, USA) maintained at 40°C. A 1-mL fraction was removed and solvent exchanged to 100 µL ethanol. The remaining 1-mL fraction was submitted for gas chromatography–mass spectrometry (GC-MS) analysis as described below.

The spiked samples were soluble exchanged to acetonitrile and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Regis Technologies, Morton Grove, IL, USA) for 4 hr at 70°C. Seven calibration standards, ranging from 15 to 1,000 ng/µL, were prepared in acetonitrile and derivatized using BSTFA under the same conditions as samples before GC-MS analysis. External standard calibration was used to generate the response factors and to generate a first-order fit to check the linearity of the calibration curve.

**Preparation of samples for Tb analysis by affinity chromatography.** This procedure was used only for July 2001 field samples. We used Randox immunoaffinity columns (Randox Laboratories) for Tb/19-nortestosterone. Phosphate buffer was added to each 10-L field sample, and samples were passed through the columns following the supplier’s protocol. A total volume of 1 L (900 mL of water sample and 100 mL phosphate buffer) was passed through the cartridge. Bound androgens were eluted with 4 mL 70% MeOH/30% water. The eluates were pooled, concentrated to 1 mL, and solvent exchanged to 100% ethanol. A 200-µL aliquot was used for A-SCREEN and E-SCREEN assays, and an 800-µL aliquot was used for HPLC separation and enzyme immunoassay (EIA).
Cell lines and culture conditions. Maintenance and propagation of estrogen-target MCF7-BOS cells (Villalobos et al. 1995) and androgen-target MCF7-AR1 cells (Szelei et al. 1997) were performed as previously described (Soto et al. 1999). Charcoal–dextran stripping of fetal bovine serum (CDFBS) was performed as previously described (Soto et al. 1991).

E-SCREEN bioassy. Human breast cancer MCF7 cells are plated into 24-well plates (Linbro, ICN Biomedical, Costa Mesa, CA, USA) at an initial density of 20,000–30,000 cells/well in 1 mL Dulbecco’s modification of Eagle’s medium (DMEM; ICN Biomedical) supplemented with 5% fetal bovine serum (HyClone, Logan, UT, USA). The cells were dispersed evenly in each well and allowed to attach for 24 hr. The seeding medium was then replaced with sample extracts diluted with 5% CDFBS in phenol red–free DMEM (Irvine Scientific, Santa Ana, CA, USA).

Each experiment includes a 17β-E2 standard dose–response curve with 15 dilutions of 17β-E2 (0.05 pM–10.0 nM) in quadruplicate wells, run simultaneously with the samples. Results from the water sample extracts are interpolated into this dose–response curve. Water extracts (100 µL in ethanol) were prepared by adding 5% CDFBS to a final volume of 10 mL and tested at five different concentrations to ensure a cell number near the half-maximal level (M50) of the 17β-E2 dose–response curve. Water blanks and T1 and T2 samples were tested at 100-, 50-, 25-, 6.25-, and 1.25-fold greater than the initial water concentration. Field samples 1–6 were first tested at the same concentrations as the water blanks. Once the range of estrogenic activity had been established, a detailed concentration curve was run within that range to accurately measure estrogenic activity. On each 24-well plate we tested five concentrations from one extract in duplicate, plus negative (5% CDFBS) and positive controls (5% CDFBS plus 100 pM 17β-E2). Assays for each water extract were repeated three to five times.

To evaluate potential cytotoxicity, each extract dilution was also tested in the presence of 100 pM 17β-E2 to induce maximal proliferation. If maximal proliferation was achieved, it was interpreted as a lack of toxicity. If the cell number was less than the positive control, it was used to qualify the results of that particular extract concentration as cytotoxic. In addition, cytotoxicity was identified through microscopic observation before fixation.

A-SCREEN bioassy. This assay uses MCF7-AR1 cells, which are stable transfectants of MCF7 cells expressing the wild-type human androgen receptor (Szelei et al. 1997). These cells proliferate maximally in 5% CDFBS and 100 pM 17β-E2, and respond to androgens by decreasing their proliferation rate. The assay compares the cell number of similar inocula of MCF7-AR1 cells grown in 5% CDFBS, 5% CDFBS plus 100 pM 17β-E2, and 5% CDFBS/100 pM 17β-E2 plus a range of concentrations of the synthetic, nonmetabolizable androgen R1881 (positive control) and a range of concentrations of a suspected androgen mimic (Soto et al. 1999). Each sample was assayed three to five times.

Field samples were analyzed by testing several dilutions of the ethanolic extract as described above for the E-SCREEN assay. The test samples, their corresponding field blanks, and an R1881 dose–response curve were processed simultaneously.

Processing for cell counting. We used fixation and staining techniques previously described by Villalobos et al. (1993). The bound sulforhodamine B (SRB) dye was solubilized using 500 µL 10 mM Tris base (pH 10.5) per well; triplicate 100-µL aliquots were transferred to 96-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) and scanned in a computerized microplate reader (Series 750; Cambridge Technology, Inc., Watertown, MA, USA) at 515 nm wavelength. The relationship between optical density and cell number was established by comparing different cell inocula and counting half of the wells with a Coulter counter (Coulter Electronics, Hialeah, FL, USA) and half via the SRB method.

Data analysis. 17β-E2 and R1881 dose–response curves were used as standards to quantify estrogenic and androgenic activity of samples in 17β-E2 equivalents (E2Eq) or R1881 equivalents (AEq). We expressed the cell number from the experiment with the 17β-E2 (or R1881) concentration relative to the maximal concentration and graphed the linearized curve. Data points with R2 > 0.85, because these points fall near the asymptotes (0.15, below detection limits; >0.85, in the plateau) where the relationship between dose and effect is meaninglessness (Feldman and Rodbard 1971). The slope and y-intercept of the linear logit-transformed dose–response curve were then used to calculate the E2Eq or AEq corresponding to B/B0 for any tested samples.

Conversion of analytical data into hormone equivalent units. M50 is the concentration producing an estrogenic (or androgenic) effect that is 50% of the maximal response. This parameter is measured to assess the potency of agonists, relative to the standard. The relative proliferative potencies (RPPs) were calculated as (M50 17β-E2 or R1881) / (M50 test chemical). Based on the mean M50, the “predicted” estrogen/androgen total load was calculated and compared with the actual values obtained by measuring bioactivity with the E-SCREEN and A-SCREEN assays.

All target analytes [estrone (E1), 17β-E2, 17α-ethinylestradiol (E2), 17β-E2, and the six resorcylic acid lactones (α-zearalanol, β-zearalanol, α-zearalenol, β-zearalenol, zearalenone, zearalanone), and MGA] were assayed using both the E-SCREEN and the A-SCREEN assays.

Immunometric detection of estrogens and androgens. Water extracts (100-µL aliquots) were diluted with purified water (Millipore-Milli-Q-PLUS Purification Pak CPMQ0041R; Millipore Corp., Billerica, MA, USA) to a final ethanol concentration of 20% and passed through 100-µg octadecyl silica gel cartridges (Bakerbond solid-phase extraction C18; J.T. Baker Inc., Phillipsburg, NJ, USA). Cartridges were washed with 2 × 1 mL methanol and equilibrated with 2 × 1 mL 20 mM Tris-HCl (pH 8.5)/methanol 80/20 (vol/vol). After sample application, the cartridge was washed twice each with 1 mL 20 mM Tris-HCl (pH 8.5)/methanol 80/20 (vol/vol) and 40% aqueous methanol. Hormones were eluted with 1 mL 80% aqueous methanol.

The solvent was evaporated at 60°C under reduced pressure (3 hPa; Uniject II, Uni Equip, Munich, Germany). For HPLC analysis, the residue was redissolved in 300 µL 20 mM Tris-acetate (pH 7.2)/acetonitrile 80/20 (vol/vol) for E2 or 300 µL purified water/methanol 80/20 (vol/vol) for Tb. According to previously established procedures (Lange et al. 2001; Schiffer et al. 2001), the extracts were processed via HPLC and prepared for analysis by specific immuno- metric methods (EIA). The estrogen and Tb fractions were analyzed by EIA as previously described (Meyer and Hoffmann 1987; Meyer et al. 1997).

GC-MS detection of anabolic agents. For low-resolution measurements of anabolic agents, we used an Agilent 5973 single-quadrupole mass spectrometer (Palo Alto, CA, USA), coupled with an Agilent 6890 gas chromatograph used with an Agilent DB-5.625 (30 mm × 0.25 mm inner diameter × 0.25 µm) analytical column (inject temperature, 275°C; injection volume, 2 µL; J&W Scientific, Folsom, CA, USA). The injection mode was pulse splitless with a 1-min purge time and a 50 mL/min purge flow. The carrier gas was helium. The oven temperature was programmed for 60°C (1 min) and 8°C/min to 310°C (5 min), and the transfer line temperature was 280°C. Temperatures were set at 150°C and 230°C for the quadrupoles and source, respectively. We used electron impact.
(EI) as the ionization technique. Mass spectra were acquired under selected ion monitoring (SIM) mode in which three to four ions per compound were scanned to enhance sensitivity.

For high-resolution measurements, the ethanolic extracts were further purified for GC–high resolution MS (GC-HRMS) through three solid-phase extraction columns (C18, diol, and silica) (Maume et al. 2001). The derivatization reagents included n-methyl-n-trimethylsilyl-trifluoroacacetamide, and trimethylsodiosilane (Fluka, Buchs, Switzerland). The measurements were obtained as described by Maume et al. (2001) using EI ionization and SIM acquisition with perfluorokerosene providing the “lock” mass and 1,3,5(10)-estratriene-16,16,17-d3β-diol providing the internal standard.

Results
Choice of extraction method. Neither 6% nor 50% ethyl ether–hexane gave acceptable recoveries. Extraction with DCM gave acceptable recoveries for all the analytes of interest: 102% for estrone (E1), 87% for 17β-E2, 99% for testosterone, 103% for Tb-17α, 100% for Tb-17β, and between 95% and 101% for six resorcylic acid lactones.

Hormonal activity of aliphatic compounds. The M50 values for the resorcylic acid lactones were as follows: α-zearalenol, 50 pM; β-zearalenol, 4.3 nM; α-zearalenol, 0.13 nM; β-zearalenol, 0.6 nM; zearalenone, 0.94 nM; and zearalanone, 0.43 nM, compared with 7.5 pM for 17β-E2. Androgens (up to 1 µM) had no estrogenic activity.

The M50 of the isomers Tb-17α and Tb-17β were 2.35 and 0.15 nM, respectively; the latter had similar potency to dihydrotestosterone (M50, 75 pM) and R1881 (M50, 68.4 pM). MGA had neither estrogenic nor androgenic activity.

Dose–response curves for 17β-E2 and R1881. The M50 values were 7.5 ± 2.1 pM for 17β-E2 (n = 9) and 68.4 ± 23.1 pM for R1881 (n = 7). The R² values for 17β-E2 (n = 9) and R1881 (n = 7) were 0.97 ± 0.04 and 0.97 ± 0.03, respectively. Figure 2 depicts the 17β-E2 and R1881 dose–response curves.

Estrogen and androgenic activity in the field samples. For June 1999 field samples, both estrogen and androgen activities were highest in site 1 samples. There was a marked decline of androgen activity in site 2 samples, comparable with that in samples from sites 3–5. Site 6 samples exhibited a lower activity, about 45–50% that of samples from sites 2–5 and about 25% that of site 1 samples; androgen activity of samples was as follows (in picomoles AEq): site 1, 9.62 ± 1.5%; site 2, 3.83 ± 14%; site 3, 4.58 ± 1%; site 4, 3.89 ± 14%; site 5, 4.58 ± 7.2%; and site 6, 2.45 ± 16%. In contrast, estrogenic activity was similar at sites 1 and 2 and roughly 50% lower at site 6 (Figure 3); estrogenic activity of samples was as follows (in picomoles E2Eq): site 1, 1.73 ± 6.2%; site 2, 2.23 ± 17%; site 3, 0.65 ± 1.5%; site 4, 0.78 ± 16%; site 5, 0.76 ± 19%; and site 6, 1.15 ± 19%

No estrogenic estrogens or androgens were found by the E-SCREEN assay that could be detected in the water blanks. Detection of estrogens and androgens by EIA and GC-MS was problematic because of the low signal-to-noise ratio. We tested for Tb-17β and its metabolites Tb-17α and TbO, as well as zearanol, 17β-E2, 17α-E2, and E1. The only analyte measurable in these conditions of extremely high noise was E1 (Table 1). There was a 6-fold difference between E1 activities at sites 1 (1,650 pg/L) and 6 (270 pg/L). E1 activity at site 3 was 354 pg/L. E1 was below detection limits in T1 and T2 samples. The detection limit for these two samples was 1–3 pg/L, two orders of magnitude below the levels found in field samples. These values were confirmed by GC-HRMS. E1-17α was detected only at site 5 (19 pg/L).

We measured the RPP of E1 (relative to 17β-E2) to assess the proportion of estrogenic activity detected in the E-SCREEN assay that is due to E1 (RPP, 0.0436). The estrogenic activity of E1 accounted for a variable portion of estrogenic activity found by the E-SCREEN assay (Table 1).

Discussion
The recent increase in the number of CAFOs raises concerns that their wastewater may contaminate downstream watercourses and thereby contribute to environmental degradation. According to the new U.S. EPA ruling (U.S. EPA 2003), more CAFOs will be required to seek discharge permits under the Clean Water Act (1970) and to develop and implement nutrient management plans.

To identify the analytes responsible for the hormonal activity, we collected samples again in September 2000. We detected both androgenic and estrogenic activity (Table 2). Detection of Tb and zearanol was thwarted because of high noise. E1 was positive (8,300 pg/L) and represented 36.34% of the total estrogenic activity detected by the E-SCREEN assay (Table 3). 17α-E2, 17β-E2, and E1 were also analyzed by GC-HRMS, yielding comparable results (Table 3). These results suggest that other estrogens in addition to our target analytes could have been present.

To decrease noise and enable the detection of Tb and its metabolites in July 2001 field samples, we chromatographed water samples taken from sites 1, 3, and 6 through Tb/19-nortestosterone immunoaffinity columns. The A-SCREEN revealed 1.37, 0.5, and 0.19 pM AEq at sites 1, 3, and 6, respectively, and no activity in the water blanks. However, the purification yield was not measured because a tracer would interfere with the bioactivity assays. As expected, the E-SCREEN assay detected no estrogenic activity in these supposedly androgen-only preparations (Table 2). Tb-17β, Tb-17α, and TbO were detected, albeit at concentrations about 100-fold lower than that needed for estrogen detection by the A-SCREEN (Table 4). The extremely low level at site 1 (theoretically the most polluted), and the low but detectable levels of Tb-17α and TbO in the water blanks suggest that these data may be artifactual.

Water blanks processed later revealed undetectable levels of the three Tb-related analytes.

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Our original purpose was to compare hormonal activities and profiles in the runoff from CAFOs that supplement their cattle with hormones than those in runoff from CAFOs that do not. We were unable to identify any sites where animals were raised without hormone supplements in a feedlot setting. In hindsight, this is not surprising because hormone supplements in a feedlot setting. In hindsight, this is not surprising because hormone supplements are given to approximately 90% of U.S. beef cattle (Balter 1999). Cattle raised without hormone supplements are usually raised at low density on open rangeland; hence, their excreta density on open rangeland; hence, their excreta hormone supplements are usually raised at low density on open rangeland; hence, their excreta density on open rangeland; hence, their excreta.

The hormones are usually given in the form of implants; the implants currently marketed contain pharmaceuticals with androgenic (testosterone, TbA), estrogenic (17β-E2, zeronol), or progestogenic (MGA) activities, and deliver either single hormones (e.g., Finaplix-H (Intervet, Millsboro, DE, USA), 200 mg TbA; Ralgro (Mallinckrodt Veterinary, Mundlein, IL, USA), 36 mg zeranol) or a mixture [e.g., Synovex-H (Fort Dodge Animal Health-Wyeth, Madison, NJ, USA), 200 mg testosterone propionate plus 20 mg E2 benzoate] (Lange et al. 2001). The progestogen agonist MGA was devoid of estrogenic and androgenic activity; however, Meyer (2001) reported that administering MGA to female cattle increases their plasma E2 levels.

We collected runoff water from feedlots where animals were treated with anabolic steroids. A significant portion of these steroids and their metabolites are excreted as conjugates (Schiffer et al. 2001), which are not extracted by DCM. However, conjugates are metabolized by bacteria into their DCM-extractable free form in sewage and surface waters (Irwin et al. 2001). Hence, the results presented here may underestimate actual exposure if conjugates were present in the water. We did not know a priori what type of hormone implant was used in this feedlot and were not able to obtain this information upon request. Therefore, we first measured the total estrogenic and androgenic activity at different points downstream of one such operation to assess the exposure to fish (Orlando et al. 2004). We then analyzed the water extracts to assess the presence of pharmaceuticals frequently used as anabolic steroids (this study).

Bioassays measure the total activity of mixtures of chemicals that act through the same receptor systems (Silva et al. 2002); activity is expressed in concentration units of the standard (Soto et al. 1997). These data are then contrasted with instrumental analyses of the sample. Ideally, the analytes measured account for the total hormonal activity found in the bioassay. The presence of additional active compounds in the mixture is suggested when the instrumental analysis reveals a lower theoretical activity than the bioassay detects.

Androgenic activity. The total androgenic activity measured by A-SCREEN seems to have originated in the feedlots, because it was highest at site 1 and decreased to < 40% at site 2, located 0.5 km downstream. The intermediate contamination site, which also drained feedlots, had androgenic activity comparable with that of site 2, which was 2-fold higher than in the reference site. At the time of sample collection, sites 4 and 5 were believed to be free of feedlot exposure. The hormonal activity in the reference sites could be a result of manure water slurry that had been sprayed on crops in the vicinity, which we learned of after the water samples had been collected, processed, and assayed.

Marginal levels of Tb and its metabolites (representing 0.1–1.1% of the total androgenic activity) were detected. Hence, the androgenic activity may be attributed to natural androgens. The octanol–water partition coefficient of natural androgens (3.3 for testosterone) suggests a potential for sorption to organic matter and, thus, a higher concentration in sediment (not analyzed) than in water.

Table 1. E1 concentration (pg/L) in water collected in July 1999, measured by EIA and GC-HRMS.

| Sample | EIA | GC-HRMS |
|--------|-----|---------|
|        | pg/L | pM^b | pg/L | pM^b |
| 1      | 1,650 | 6.1 | 0.265 | 15.36 |
| 2      | < 530 | 1.3 | 0.037 | 1.0 |
| 3      | 370   | 1.4 | 0.060 | 9.10 |
| 4      | < 210 | 1.0 | 0.172 | 28.21 |
| 5      | 1,600 | 5.9 | 0.235 | 33.95 |
| 6      | 270   | 1.0 | 0.053 | 4.64 |
| T1     | < 5   | 0.22 | 0.010 |
| T2     | < 1   | 0.24 |
| Blank  | < 1   | 5.64 |
| Spike  | NA^a  | 16.8 |

NA, not assayed.

Table 2. Total androgen and estrogen activity in water samples collected during 2000 and 2001.

| Sample | AEq (pM) | E2Eq (pM)^d | E2Eq (%)^d |
|--------|----------|-------------|-------------|
| September 2000 | | | |
| Site 1 | 3.75 ± 11.5% | Toxic/ND^c | | |
| Site 6 | 0.54 | ND | | |
| June 2001 | | | |
| Site 1 | 1.03 | ND | | |
| Site 3 | 0.19 | ND | | |
| Blank 1 | 0.19 | ND | | |
| Blank 2 | 0.19 | ND | | |
| Spike | 56.42 | ND | | |

Table 3. Estrogen levels (pg/L) in water samples collected in September 2000 as detected by EIA and GC-HRMS.

| Site | E1 | 17α-E2 | 17β-E2 |
|------|----|--------|--------|
|     |    | EIA    | GC-MS  | EIA    | GC-MS  |
| 1   | 8,300 | 7,882 | < 3,800 | ND | < 3,200 | ND |
| 6   | 900  | 2,434 | 35       | 26 | 84     | ND |

ND, not detectable.

Table 4. Androgen levels (pg/L) in water samples collected in July 2001 as determined by EIA^a.

| Sample | Tb-17β | Tb-17α | Tb0 |
|--------|--------|--------|-----|
| Site 1 | 1.5    | 5.4    | 7.6 |
| Site 3 | 1.3    | 35     | 16  |
| Site 6 | < 0.4  | 1.6    | 1.9 |
| Blank 1 | 0.3 | 1.6 | 0.3 |
| Blank 2 | 0.3  | 1.6 | 0.3 |

^aAfter cleanup with immunoaffinity columns for Tb19-nortestosterone.
Resorcylic acid lactones were not detected; instead, E2 was detected in all sites and represented up to 46% of the total estrogen activity. 17α-E2 and 17β-E2 were barely detectable in some samples. The main metabolite of 17β-E2 in cattle excreta was 17α-E2. We were not surprised, however, to find that the main estrogen detected was E1. It has been established that microorganisms in the environment degrade estrogens and that E2 is rapidly transformed to E1 in river water and in sediments (Jürgens et al. 2002). The log octanol–water partition coefficients of E2 and E1 are reported to be in the range of 3–4, indicating sorption potential to organic matter. Therefore, higher concentrations of estrogens may be present in the sediment. This is especially important concerning E1, which is persistent (Environment Agency 2002). The fact that E1 did not present up to 46% of the total estrogenic activity. Instead, E1 was detected in all sites and represented 0.8–4.0% of the total estrogen activity found at these two sites may be due to the release of these chemicals into water bodies in the United States and Europe with numerous bioactive agents, including natural hormones, xenohormones, and pharmacologic agents (Allen et al. 1999; Kolpin et al. 2002; Vethaak et al. 2002), and point to the urgent need to take steps to curtail environmental degradation caused by the release of these chemicals into water bodies.

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