

INTEGRATED ASSESSMENT OF RUNOFF FROM LIVESTOCK FARMING OPERATIONS:
ANALYTICAL CHEMISTRY, IN VITRO BIOASSAYS, AND IN VIVO FISH EXPOSURES

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Abstract: Animal waste from livestock farming operations can contain varying levels of natural and synthetic androgens and/or estrogens, which can contaminate surrounding waterways. In the present study, surface stream water was collected from 6 basins containing livestock farming operations. Aqueous concentrations of 12 hormones were determined via chemical analyses. Relative androgenic and estrogenic activity was measured using in vitro cell assays (MDA-kb2 and T47D-Kbluc assays, respectively). In parallel, 48-h static-renewal in vivo exposures were conducted to examine potential endocrine-disrupting effects in fathead minnows. Mature fish were exposed to surface water dilutions (0%, 25%, 50%, and 100%) and 10-ng/L of 17 α -ethynylestradiol or 50-ng/L of 17 β -trenbolone as positive controls. Hepatic expression of vitellogenin and estrogen receptor α mRNA, gonadal ex vivo testosterone and 17 β -estradiol production, and plasma vitellogenin concentrations were examined. Potentially estrogenic and androgenic steroids were detected at low nanogram per liter concentrations. In vitro estrogenic activity was detected in all samples, whereas androgenic activity was detected in only 1 sample. In vivo exposures to the surface water had no significant dose-dependent effect on any of the biological endpoints, with the exception of increased male testosterone production in 1 exposure. The present study, which combines analytical chemistry measurements, in vitro bioassays, and in vivo fish exposures, highlights the integrated value and future use of a combination of techniques to obtain a comprehensive characterization of an environmental chemical mixture. *Environ Toxicol Chem* 2014;33:1849–1857. Published 2014 Wiley Periodicals, Inc. This article is a US Government work and, as such, is in the public domain in the United States of America.

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INTRODUCTION

Animal waste runoff contributes excess nutrients, bacteria, and, of particular concern in the present study, hormones to surrounding waterways [1–3]. Specifically, depending on the livestock type and management practices, animal waste can contain varying levels of natural and synthetic androgens or estrogens, which can be transported to nearby aquatic systems through runoff from feedlots, manure lagoons, or fields where manure has been applied as fertilizer. Naturally occurring sex steroids, such as 17 β -estradiol (E2), estrone, and testosterone (T), can persist for extended periods of time in animal wastes [4,5]. In addition to being a concentrated source of these endogenous steroids, many operations utilize synthetic steroid hormones to promote rapid growth of lean tissue [6,7]. Consequently, after rainfall events, there is potential for the surrounding waterways of feedlot basins to contain complex mixtures of natural and/or synthetic endocrine-active chemicals that might negatively impact exposed aquatic biota.

When assessing the potential risks associated with exposure to endocrine-active chemical mixtures in the environment (e.g., livestock waste runoff), combinations of analytical chemistry techniques, in vitro bioassays, and in vivo assays might be more useful for predicting responses than a single approach [8,9]. For example, specific chemicals can be quantified in environmental water samples using analytical chemistry techniques. While these techniques may prove useful in partially defining the composition of a chemical mixture, they can be limited by factors such as inadequate detection limits and the ability to detect only those chemicals for which methods and standards are available. In vitro cell bioassays offer a relatively sensitive and efficient method of characterizing the integrated estrogenic and/or androgenic activity within complex environmental samples that contain multiple interacting chemicals. For example, the estrogen-responsive T47D-KBluc in vitro cell assay has proven to be a useful tool for characterizing the estrogenicity of complex environmental samples as well as predicting molecular responses in fish [10]. Similarly, the androgen-responsive MDA-kb2 in vitro cell line can be used to reliably estimate the androgenicity of environmental mixtures [8]. However, such assays cannot identify the specific chemicals causing the activity and, hence, do not provide sufficient information needed to develop contaminant mitigation options. Furthermore, in vitro

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assays represent highly simplified biological systems that may not adequately capture the range of toxicokinetic and toxicodynamic variables that can influence outcomes *in vivo*. Thus, while they provide important insights into hazard potential, predicting risk from *in vitro* bioassay results alone remains challenging. As a consequence, strategic deployment of integrated approaches that combine the complementary strengths of analytical chemistry, *in vitro* bioassays, and *in vivo* exposure testing has been advocated for environmental assessment [9,11,12].

As a case study to evaluate this integrated approach, surface water samples from 6 basins containing livestock farming operations were collected following rainfall events and analyzed using a combination of analytical chemical measurements, *in vitro* cell bioassays, and *in vivo* fish exposures. These samples were strategically selected from a national-scale study of livestock farming operation basins being conducted by the US Geological Survey (USGS) [13]. Specifically, we estimated relative androgenic and estrogenic activity of stream water collected from select basins using 2 well-characterized recombinant human *in vitro* cell bioassays. The *in vitro* assays were paired with 48-h static-renewal *in vivo* exposures to examine the endocrine activity of the samples in male and female fathead minnows. The purpose of pairing the *in vitro* and *in vivo* assays was to evaluate whether *in vitro* assays are useful tools for predicting the relative potencies and effects these mixtures might elicit in exposed fish. Additionally, concentrations of select chemicals in the runoff surface water were quantified using analytical chemistry methods. The objective of the present case study was to apply an integrated strategy utilizing analytical chemistry, *in vitro* bioassays, and *in vivo* exposures to assess the potential impact of complex mixtures of endocrine-active chemicals from livestock farming operation waste runoff.

MATERIALS AND METHODS

Runoff water sample collection

Stream water was collected as grab water samples from the centroid of flow from 6 livestock farming operation basins within the United States following manure application or rainfall runoff events. The sites represented dairy, cattle, and poultry operations at sites in Wisconsin, Iowa, New York, Arkansas, and Kentucky, USA (Supplemental Data, Table S1). Water samples were collected by USGS personnel as part of a larger study examining the influence of small watersheds with homogenous animal types on multiple aspects of stream water and sediment quality [13]. Water sample collections were conducted according to USGS water-collection guidelines [14]. A field quality-assurance protocol was used to determine the effect, if any, of field equipment and procedures on the concentrations of hormones in water samples. Field blanks, made from laboratory-grade organic free water, were submitted 5 times from the 6 livestock farming operation basins and analyzed for all target compounds. Field blanks were subjected to the same sample processing, handling, and equipment as the stream samples. No detections of the target compounds occurred in any of the field blanks collected. In addition, a field replicate was collected at 1 of the 6 sites to quantify the reproducibility of the data. Field duplicates were water samples collected along with the regular environmental sample and processed as if they had been obtained at a unique site. The presence or absence of the target compounds was confirmed in 100% of the chemical determinations.

Approximately 75 L of water was collected at each location for use in *in vivo* fish exposures and *in vitro* cell bioassays. The water samples were collected into low-density polyethylene containers, previously rinsed with high-quality deionized water. Samples were transported on ice overnight to the US Environmental Protection Agency (USEPA), Mid-Continent Ecology Division (MED) in Duluth, Minnesota. Water sample aliquots were also shipped to the USEPA Toxicity Assessment Division in Research Triangle Park (RTP), North Carolina, for an interlaboratory *in vitro* cell assay comparison study, described in the *In vitro bioassays* section. Simultaneously, additional water samples were collected by the USGS and allocated for chemical analyses by a USGS laboratory [15] and by researchers at the University of Nevada–Reno [16,17]. Samples for these labs were placed in 500-mL high-density polyethylene bottles, shipped on ice overnight, and processed immediately on receipt or stored at -25°C until processed. The analytes determined in unfiltered water samples by the USGS lab were predominantly steroids (or precursors) and included cholesterol, coprostanol, estrone, estriol, E2, 17α -estradiol, *cis*-androsterone, 4-androstene-3,17-dione, and epitestosterone. Analytes determined in filtered (0.7- μm nominal pore size glass-fiber filter) water samples by the University of Nevada–Reno included 17α -trenbolone, 17β -trenbolone (TRB), and trendione. The analytical methods were performed as previously described [15–17]. Basic water-quality measurements including pH, conductivity, dissolved oxygen, and water temperature also were recorded at the time of sample collection (Supplemental Data, Table S1).

In vitro bioassays

The estrogen-responsive T47D-KBluc and androgen-responsive MDA-kb2 cell lines were used to assess the estrogenicity and androgenicity of the water samples [18,19]. Cell culture medium was directly prepared from ambient water samples, based on a previous study where this method was found to be a more effective and consistent approach than using a concentrated solvent extract [10]. Therefore, on receipt, each environmental water sample was immediately used to prepare exposure media suitable for each cell assay. Sample preparation for the T47D-KBluc assays consisted of combining 20 mL of sample water with 0.21 g of powdered RPMI 1640 medium (catalog number 060M8310; Sigma), 0.05 g D-(+)-glucose (catalog number G5400; Sigma), 0.03 g sodium bicarbonate (catalog number S5761; Sigma), 200 μL *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (1M, catalog number BP299-1; Fisher Scientific), 200 μL sodium pyruvate (100 mM, catalog number 11360-070; GIBCO), and 1.0 mL charcoal dextran-treated fetal bovine serum (catalog number SH30068.03; HyClone). Sample preparation for the MDA-kb2 assay consisted of combining 20 mL of sample water with 0.27 g Leibovitz's L-15 (catalog number 41300-021; GIBCO), 220 μL Anti/Anti (100 \times , catalog number 15240; GIBCO), and 2.0 mL fetal bovine serum (catalog number SH30071.03; HyClone). After preparing media from the ambient water, the samples were sterilized by filtration using a syringe-driven Luer-lock filter unit (Henke Sass Wolf) with a 0.22- μm pore size polyethersulfone membrane filter attachment (Millipore), placed in 50-mL centrifuge tubes, and stored at -80°C until used in the cell bioassays.

For the T47D-KBluc assay, cells were removed from culture flasks, counted, and diluted to 100 000 cells/mL in assay media (RPMI 1640 medium [GIBCO] supplemented with 5% charcoal dextran-filtered fetal bovine serum, prepared using Millipore water). Then, 100 μL of the cell solution was seeded into clear-

bottomed, opaque-sided, 96-well plates (catalog number 165306; Thermo Scientific) for a final density of 10 000 cells per well. The plates were incubated at 37 °C and 5% CO₂ in a humidified atmosphere for 24 h. Following the initial incubation to allow for cell attachment, cells were dosed with 17 α -ethynylestradiol (EE2), which was 2-fold serially diluted in assay media with 9 concentrations ranging from 16.47 ng/L to 0.06 ng/L, using ethanol as the carrier solvent. Ethanol and assay media controls were also included on each plate, with the solvent never exceeding 0.1% per well. Frozen environmental samples were thawed, pH-adjusted to 7.3, and sterilized by filtration again using a 0.22- μ m pore size sterile syringe filter as described above. The environmental media sample was serially diluted in a 50% series using RPMI assay media to produce concentrations ranging from 100% to 12.5% environmental media sample. All samples, standards, and controls were run in duplicate or triplicate on each plate, with 100 μ L of each solution added to wells containing cells and incubated for an additional 16 h to 24 h at 37 °C under 5% CO₂. The mean (\pm standard deviation [SD]) coefficient of variation (CV) values for the T47-KBluc assays were 7.4 \pm 4.3% for the ethanol controls, 18.77 \pm 7.52% for the assay media controls, and 10.5 \pm 7.0% for the environmental samples.

For the MDA-kb2 assay, cells were removed from culture flasks, counted, and diluted to 500 000 cells/mL in assay media (Leibovitz's L-15 [GIBCO] supplemented with 10% fetal bovine serum and 1.1 \times Anti/Anti, prepared using Millipore water). Then, 100 μ L of the cell solution was seeded into clear-bottomed, opaque-sided, 96-well plates (Thermo Scientific) for a final density of 50 000 cells per well. The plates were incubated at 37 °C for 4 h. Following the initial incubation, cells were dosed with TRB, which was 2-fold serially diluted in supplemented L-15 medium with 10 concentrations ranging from 1350 ng/L to 2.6 ng/L, using ethanol as the carrier solvent. Ethanol and assay media controls were also included on each plate, with the solvent never exceeding 0.1% per well. The frozen environmental sample was thawed, pH-adjusted to 7.6, and sterilized by filtration as described above. The environmental media sample was diluted in a 50% series using supplemented L-15 medium to produce concentrations ranging from 100% to 3.13% environmental media sample. All samples, standards, and controls were run in duplicate or triplicate on each plate, depending on space, with 100 μ L of each solution added to wells containing cells and incubated for an additional 16 h to 24 h at 37 °C. The CV values for the MDA-kb2 assays were 8.05 \pm 3.67% (mean \pm SD) for the media controls, 7.95 \pm 4.32% for the ethanol controls, and 6.54 \pm 4.60% for the samples.

After exposure to the standard, sample, or control solutions, cytotoxicity was analyzed using a live/dead assay (catalog number L3224; Invitrogen) according to the manufacturer's protocol. Following the cytotoxicity test, a luminescence assay was conducted, which consisted of adding 25 μ L of D-luciferin (1.0 mM in Millipore water; catalog number E1605; Promega) and 25 μ L of reaction buffer to each well via direct injection in a luminometer plate reader (Biotek, Synergy 4; Gen 5 Software) [8,10]. The reaction buffer consisted of 45 mL Millipore water, 1.25 mL of 1M glycylglycine (catalog number BP1304; Fisher), 0.75 mL of MgCl₂ (catalog number M8266; Sigma), 2.5 mL of 100 mM adenosine 5'-triphosphate disodium salt (catalog number A6419; Sigma), and 0.5 mL of 50 mg/mL bovine serum albumin (catalog number A4161; Sigma). Luminescence was recorded in relative luminescence units. Estrogenicity and androgenicity of the samples were estimated relative to the EE2 and TRB standard curves, respectively.

In addition to the T47D-KBluc and MDA-kb2 bioassays conducted at the MED laboratory, the water samples were analyzed with the T47D-KBluc assay at the RTP laboratory for an interlaboratory comparison. The assay conducted at RTP used similar methods to those of the MED laboratory but used E2 standards (range, 27.24–0.0027 ng/L) rather than EE2 standards [18]. All ethanol controls and samples were run in quadruplicate in the assays conducted at RTP. The CV values were 26.0 \pm 21.3% (mean \pm SD) for the ethanol controls and 18.1 \pm 12.8% for the environmental samples.

In vivo exposures

Immediately on sample receipt, 48-h static-renewal exposures to the livestock farming operation runoff water were conducted using fathead minnows (*Pimephales promelas*). Sexually mature male and female fathead minnows, 5 mo to 6 mo of age, were obtained from an on-site culture unit at the MED laboratory. All laboratory procedures involving animals were reviewed and approved by the MED Animal Care and Use Committee, in accordance with the US Animal Welfare Act and Interagency Research Animal Committee guidelines [20]. The static-renewal exposure treatments included a Lake Superior Water (sand-filtered, ultraviolet-treated) control, and 3 dilutions of the livestock farming operation runoff water in Lake Superior water (25%, 50%, and 100% livestock farming operation water sample). Water volumes in the 20-L glass test tanks were 10 L. Each treatment tank held 4 male and 4 female fish. Two replicate tanks were used at each treatment level to obtain a total sample size of 8 males and 8 females per treatment. In addition, an estrogen positive control of EE2 and an androgen positive control of TRB were included with each exposure. A stock solution in deionized water was prepared for both EE2 and TRB, which was subsequently diluted with Lake Superior water to obtain nominal treatment concentrations of 10 ng/L EE2 and 50 ng/L TRB in the 10-L test solutions. Tanks were aerated using air stones with a constant air flow rate and held in a 25 \pm 1 °C water bath with a 16:8 light:dark photoperiod. Fish were fed adult brine shrimp (San Francisco Bay Brand) twice daily. One hundred percent of the tank water was exchanged 24 h after exposure initiation. During the renewal, fish were transferred and temporarily held in buckets containing tank water from corresponding exposure treatments (~10 min).

After the 48-h exposure, fish were anesthetized in buffered tricaine methanesulfonate (MS-222, Finquel; Argent), and wet weights were recorded. Blood was collected from the caudal vasculature and centrifuged in heparinized microhematocrit tubes to isolate the plasma, which was then stored at –80 °C until vitellogenin (VTG) protein was measured. The liver was removed, snap-frozen in liquid nitrogen, and stored at –80 °C until RNA was extracted for gene expression analysis. A portion of each gonad (~10–20 mg) was collected for use in an ex vivo steroidogenesis assay. The remaining gonad tissue was preserved for future RNA extraction. To prevent degradation by RNases or cross-contamination, dissection tools were cleaned with RNaseZap (Ambion) between each sample.

Gene expression analyses

Quantitative real-time polymerase chain reaction assays (qRT-PCRs) were conducted to determine the relative abundance of estrogen receptor α (*esr1*) and vitellogenin (*vtg*) mRNA transcripts. Total RNA from male liver samples was extracted using TRI Reagent[®] (Sigma) according to the manufacturer's protocol. A Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies; Thermo Scientific) was used to determine RNA

concentration and quality. Total RNA samples were then diluted to a uniform concentration of 10 ng/ μ L RNA before the qRT-PCR analyses. The qRT-PCRs were performed using Power SYBR Green RNA-to-Ct 1-Step Kits (Applied Biosystems) according to the manufacturer's protocol. Each 12- μ L reaction mixture contained 20 ng total RNA, 200 nM forward primer, and 200 nM reverse primer. The primers used for measurement of fathead minnow *esr1* (5'-CACCCACCAGCCCTCAG-3'/5'-CACCTCACACAGACCAACAC-3') were described previously by Filby and Tyler [21], and the fathead minnow 5' region *vtg* primers (5'-CACAATCCCAGCTCTGCGTGA-3'/5'-TGGCCTCTGCAGCAATATCAT-3') were developed by Biales et al. [22]. Using a 7500 real-time PCR system (Applied Biosystems), the thermocycling program was set to 48 °C for 30 min for the reverse transcription step, followed by 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min for PCR amplification, and finally a dissociation stage of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Relative transcript abundance of each target gene was quantified relative to a gene-specific complementary DNA (cDNA) standard curve following a 10-fold dilution series with 6 concentrations. The cDNA standards were prepared as described by Villeneuve et al. [23]; however, a second round of amplification for preparing an RNA standard was not conducted. The qRT-PCR amplification efficiencies ranged from 76% to 100% for *esr1* analyses and from 94% to 107% for *vtg* analyses.

Steroid and vitellogenin analyses

Using a method adapted from McMaster et al. [24], *ex vivo* production of T from testis and ovary tissue and E2 from ovary tissue was determined using a radioimmunoassay as previously described [25]. Plasma VTG protein concentrations were measured by enzyme-linked immunosorbent assay as previously described [26,27] using a polyclonal antibody and a purified fathead minnow VTG standard.

Statistical analyses

Data from the T47D-KBluc and MDA-kb2 cell bioassays were used to estimate total estrogenic equivalents (EEQs) and TRB equivalents (TRB-EQs), respectively, in the surface water samples. The estrogenicity of the samples was determined relative to EE2 standard curves at the MED and relative to E2 standard curves at RTP, and androgenic activity was determined relative to TRB. The calculation methods used to estimate EEQs and TRB-EQs are similar to those described by Alvarez et al. [13]. For each plate, fold-induction of each standard and sample was calculated as a function of the average relative luminescence units measured for the ethanol control. The standard curve concentrations and the fold-induction replicate data were log-transformed to correct for heterogeneity of variance. The median effect concentration (EC50), Hill slope, and Top best fit value of the standard curve were determined using a nonlinear regression (log agonist vs response-variable slope; GraphPad Prism 5.02; GraphPad Software) of the transformed fold-induction data, constraining the bottom of the standard curve to 0. The percentage of the maximum EE2 and TRB responses for the T47D-Kbluc and MDA-kb2 assays, respectively, were calculated by dividing the transformed fold-induction replicate data by the Top best fit value and multiplying by 100. Estrogenic and androgenic activities of the surface water samples were determined by calculating the EC50 of the sample dilution curve using a nonlinear regression (log agonist vs response-variable slope), while constraining the bottom to 0, the top to 100, and the slope to that generated by the standard

curve. This constrains the data in a manner consistent with the assumptions of relative potency estimation (i.e., equal efficacy and parallel slopes [28,29]). Estrogenic equivalents and TRB equivalents were calculated by dividing the standard curve EC50 by the sample dilution that produced a 50% response. EEQs and TRB-EQs are reported in nanograms per liter. The estrogenicity and androgenicity of each water sample also were determined using the mean response as a percentage of the maximum response (generated by the EE2 or TRB standard curves, respectively). The significant response levels for each assay were defined as 3 SD above the mean luminescence values for the solvent controls and media controls. These mean significant response levels were approximately 0.1 ng/L EE2-EQ for the T47D-Kbluc assays and approximately 3.5 ng/L TRB-EQ for the MDA-kb2 assays.

To directly compare equivalency results between the MED and RTP labs, which used different chemical standard curves (i.e., EE2 and E2, respectively), a direct comparison of EE2 and E2 was conducted. Two plates containing EE2 and E2 triplicate standard curves and ethanol controls were run to facilitate this chemical potency comparison. The EC50 values for the 2 separate EE2 standard curves were calculated using a nonlinear regression (log agonist vs response-Find EC50). The EE2 EC50 was 2.5 ng/L with a 95% confidence interval of 2.1 ng/L to 2.8 ng/L. The E2 EC50 was 1.7 ng/L with a 95% confidence interval of 1.5 ng/L to 2.0 ng/L. The equivalency factor of the 2 chemicals was calculated by dividing the E2 EC50 by the EE2 EC50. The results indicated a modest difference between the potency of these 2 chemicals in the T47D-Kbluc assay; consequently, the sample E2-EQs from RTP were multiplied by the equivalency factor of 0.7 to make direct comparisons to the sample EE2-EQs determined at the MED.

Data normality for the steroid and VTG protein measurements and the qRT-PCR results was assessed using a Kolmogorov-Smirnov test. The qRT-PCR data were log₁₀-transformed to meet parametric assumptions. Parametric data were analyzed using one-way analysis of variance to test for differences across treatment groups. Duncan's multiple-comparisons test was used as a post hoc test to determine significant differences between treatment groups. When VTG protein concentrations were below the assay detection limit, values of one-half the detection limit were used in the analysis. Differences for all statistical tests were considered significant at $p < 0.05$. Statistical analyses were conducted using Statistica 10 (StatSoft) and GraphPad Prism v. 5.02.

RESULTS

Analytical chemistry

Water samples from the Wisconsin dairy, Wisconsin cattle grazing, and New York dairy livestock farming operation basins contained detectable levels of several targeted chemicals, including cholesterol, coprostanol, estrone, 17 α -estradiol, *cis*-androsterone, 4-androstene-3,17-dione, and epitestosterone (Table 1). Of the 12 analytes measured, only cholesterol was detected in the water samples from either of the poultry basins, which could be a result of no manure application on fields at these sites. Cholesterol in the 6 water samples ranged from 319 ng/L to 15 900 ng/L. Steroid analytes were detected only in water samples containing at least 2000 ng/L cholesterol, except for TRB in the Iowa cattle basin. Coprostanol, estrone, and 17 α -estradiol concentrations were generally correlated with cholesterol concentrations. In contrast, the concentrations of *cis*-androsterone and 4-androstene-3,17-dione were greater in

Table 1. Concentrations (ng/L) of hormones detected in the unfiltered or filtered (17 α - and 17 β -trenbolone and trenbolone measurements only) surface water samples collected from 6 livestock farming operation basins^a

Analyte	RL (ng/L)	WI dairy	WI cattle	IA cattle	NY dairy	AR poultry	KY poultry
Cholesterol	200	5230	2420	319	15 900	558	1390
Coprostanol	200	1616	432	<200	7050	<200	<200
Estrone	0.8	3.5	0.8	<0.8	8.1	<0.8	<0.8
Estriol	2.0	<2.0	0.3 ^b	<2.0	<2.0	<2.0	<2.0
17 β -estradiol	0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8
17 α -estradiol	0.8	2.1	1.0	<0.8	3.8	<0.8	<0.8
cis-androsterone	0.8	2.2	1.0	<0.8	0.7	<0.8	<0.8
4-androstene-3,17-dione	0.8	2.9	0.9	<0.8	1.8	<0.8	<0.8
Epitestosterone	4.0	1.2 ^b	0.4 ^b	<4.0	1.5 ^b	<4.0	<4.0
17 α -trenbolone	3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0
17 β -trenbolone	3.0	<3.0	<3.0	3.0	<3.0	<3.0	<3.0
Trenbolone	3.0	<3.0	<3.0	<3.0	6.0	<3.0	<3.0

^a See Alvarez et al. [13] for details on sample collection and chemical analyses. Estrone, estriol, 17 β -estradiol, and 17 α -estradiol data also reported in Alvarez et al. [13].

^b Concentration qualified as estimated because less than reporting level.

RL = reporting level. WI = Wisconsin; IA = Iowa; NY = New York; AR = Arkansas; KY = Kentucky.

water from the Wisconsin dairy basin than the New York dairy basin, despite the fact that greater cholesterol concentrations were detected in the New York dairy basin.

In vitro bioassays

All samples elicited estrogenic responses in the T47D-Kbluc assays conducted at the MED. Water from the dairy basins elicited the strongest estrogenic responses, whereas water from the Kentucky poultry basin (which had no manure applied) produced the weakest response (Figure 1). The interlaboratory comparison of the T47D-Kbluc assays conducted at the MED and RTP produced relatively consistent results despite the different chemical standards used. Although it is not appropriate to statistically compare the relative estrogenic equivalent estimates directly between the 2 laboratories because of the assumptions inherent in estrogenic equivalent estimation

(i.e., parallel slopes between sample and standard curve, equal efficiency), the rank order of estrogenicity among the sites is relatively similar between the MED and RTP. Both labs produced data indicating that the New York dairy sample produced the strongest estrogenic response, followed by the Wisconsin dairy sample, whereas the samples with weaker estrogenic responses did not match in rank order between the labs (Table 2).

Results from the MDA-kb2 assays conducted at the MED indicated weak androgenicity in the New York dairy water sample, producing a response that was $18.11 \pm 1.53\%$ of the maximum TRB response (Figure 2). The androgenicity of all other livestock farming operation water samples fell below the assay mean significant response level. None of the samples produced cytotoxicity in the cells (data not shown).

In vivo exposures

As anticipated, relative hepatic transcript abundance of the estrogen-responsive genes *vtg* and *esr1* was significantly upregulated in male fathead minnows exposed to 10 ng/L EE2 in all 6 of the *in vivo* studies (Figure 3; Supplemental Data, Figure S1). Hepatic *esr1* mRNA abundance was significantly upregulated in male fish exposed to the 25% and 100% treatments for the Wisconsin cattle operation exposure and significantly downregulated in male fish exposed to the 50% treatment in the Kentucky poultry operation exposure (Figure 3). Significant upregulation of *vtg* mRNA expression in male livers was also observed in the Kentucky poultry operation samples, although the response was not concentration-dependent (Figure 3). No significant treatment effects on hepatic *vtg* and *esr1* relative transcript abundance, compared with controls, were observed for male fish exposed to the Wisconsin dairy, Iowa cattle, New York dairy, or Arkansas poultry operation samples (data not shown). Vitellogenin protein was measured in male plasma from the New York dairy operation, a site that showed relatively high estrogenic activity *in vitro*, and the Kentucky poultry operation, a site that showed little estrogenicity in the T47D-Kbluc assay. Plasma VTG concentrations were significantly increased in males exposed to EE2 in both studies but not significantly increased in males exposed to the livestock farming operation runoff water *in vivo* (Figure 4). Gonadal *ex vivo* production of T and E2 was not significantly affected in female fish exposed to the livestock farming operation treatments in any study (Supplemental Data, Figure S2). Similarly, male gonadal

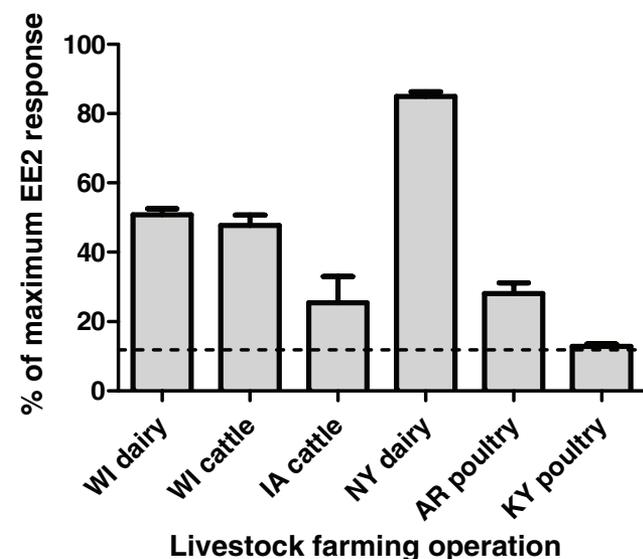


Figure 1. Estrogenic activity of surface water runoff samples from 6 livestock farming operation basins. Bars on graph represent mean (\pm standard error; $n = 3$) expressed as a percentage of the maximum 17 α -ethynylestradiol (EE2) response in the T47D-Kbluc cell bioassays conducted at the Mid-Century Ecology Division. The dashed line indicates the significant response level, which is 3 standard deviations above the mean control media response. WI = Wisconsin; IA = Iowa; NY = New York; AR = Arkansas; KY = Kentucky.

Table 2. Comparison of estimated estrogenic activity of surface water samples collected from 6 livestock farming operation basins determined using T47D-Kbluc assays at the US Environmental Protection Agency, Mid-Continent Ecology Division (MED), Duluth, Minnesota, and the Toxicity Assessment Division, Research Triangle Park (RTP), North Carolina^a

Site	MED	RTP	RTP
	EE2 equivalent (ng/L) (95% CI)	E2 equivalent (ng/L) (95% CI)	Adjusted to EE2 equivalent (ng/L) (95% CI)
WI dairy	0.71 (0.61–0.83)	1.05 (0.82–1.22)	0.74 (0.57–0.85)
WI cattle	0.65 (0.52–0.81)	0.13 (0.10–0.18)	0.09 (0.07–0.13)
IA cattle	0.29 (0.21–0.41)	0.50 (0.45–0.56)	0.35 (0.32–0.39)
NY dairy	2.47 (2.23–2.73)	4.72 (3.98–5.60)	3.30 (2.79–3.92)
AR poultry	0.35 (0.29–0.41)	0.23 (0.17–0.31)	0.16 (0.12–0.22)
KY poultry	0.21 (0.11–0.38)	0.41 (0.33–0.50)	0.29 (0.23–0.35)

^a Values are expressed as estimates with 95% confidence interval (CI). Research Triangle Park equivalents were adjusted by the 0.7 equivalency factor to account for the 17 α -ethynylestradiol (EE2) and 17 β -estradiol (E2) potency differences.

WI = Wisconsin; IA = Iowa; NY = New York; AR = Arkansas; KY = Kentucky.

ex vivo T production was not significantly affected by livestock farming operation treatment (Supplemental Data, Figure S3), with the exception of males exposed to the 100% Wisconsin cattle operation treatment, which elicited a concentration-dependent increase in ex vivo T production (Figure 5).

DISCUSSION

The overall goal of the present case study was to apply an integrated strategy employing analytical chemistry, in vitro bioassays, and in vivo fish exposures to assess 6 environmental water samples. By utilizing a combination of these approaches, we were able to determine that 1 procedure alone would be insufficient to characterize these environmental water samples. Although each approach has important strengths and value, when taken alone they give a slightly different perspective on the composition and potential effects associated with exposure to samples. Therefore, to fully understand the potential impact of environmental water samples on biota, these techniques are best examined collectively.

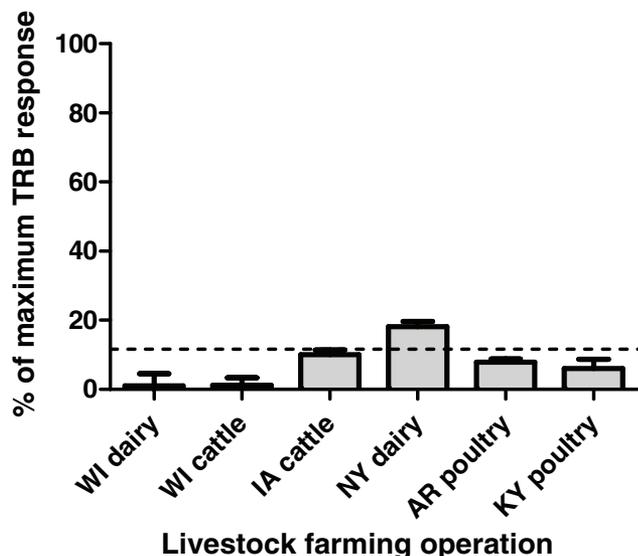


Figure 2. Androgenic activity of surface water runoff samples from 6 livestock farming operation basins. Bars on graph represent mean (\pm standard error; $n = 3$) expressed as a percentage of the maximum 17 β -trenbolone (TRB) response in the MDA-kb2 cell bioassays. The dashed line indicates the significant response level, which is 3 standard deviations above the mean control media response. WI = Wisconsin; IA = Iowa; NY = New York; AR = Arkansas; KY = Kentucky.

Analytical chemistry and in vitro bioassay comparisons

Of the 6 livestock farming operation runoff samples collected, the Wisconsin and New York dairy basins had the highest stream flow and were the basins with the largest drainage area (Supplemental Data, Table 1). The analytical chemistry results (Table 1) indicate that the 2 samples with the highest measured concentrations of coprostanol, a marker of fecal matter [30], were from the 2 dairy basins. Water collected from these dairy basins, as well as the Wisconsin cattle basin, were the only samples that contained detectable concentrations of the endogenous estrogens, estrone and 17 α -estradiol. Consistent with this, the dairy basin samples also elicited the strongest in vitro estrogenic response in the estrogen-responsive T47D-KBluc cell assays conducted at MED and RTP (Figure 1 and Table 2). Although no measureable amounts of the targeted estrogens were detected in water samples from the other sites, estrogenic activity was nonetheless detected using the T47D-KBluc assays. One possible explanation for the observed estrogenic response in the cell assays may be lower significant response levels (~ 0.1 ng/L EE2 equivalent) than the analytical reporting levels (Table 1). An alternative explanation might be that several estrogens are present at levels below the analytical detection limit and have an additive effect resulting in an estrogenic in vitro response [31]. Finally, it is possible that "unidentified" (and, hence, unmeasured) estrogenic chemicals were present in the samples. In any instance, data from the present study illustrate the importance of both chemical analyses and in vitro bioassay screening for characterization of water samples.

Little androgenic activity was observed in the MDA-kb2 cell assays, with 5 of the 6 samples below the average assay significant response level of approximately 3.0 ng/L TRB equivalent (Figure 2). Synthetic androgens, TRB (Iowa cattle) and trenbolone (New York dairy), were observed; and similarly low concentrations of 3 natural androgens, *cis*-androsterone, 4-androstene-3,17-dione, and epitestosterone (estimated concentration below the reporting level), were detected in water from the Wisconsin cattle and Wisconsin and New York dairy basins (Table 1). Based on the frequency and magnitude of detection of steroidal androgens, it is not surprising that little to no androgenic activity was associated with the livestock farming operation samples in the MDA-kb2 assay.

In vivo response

Another study objective was to compare the analytical chemistry and in vitro bioassay results with data from in vivo fish

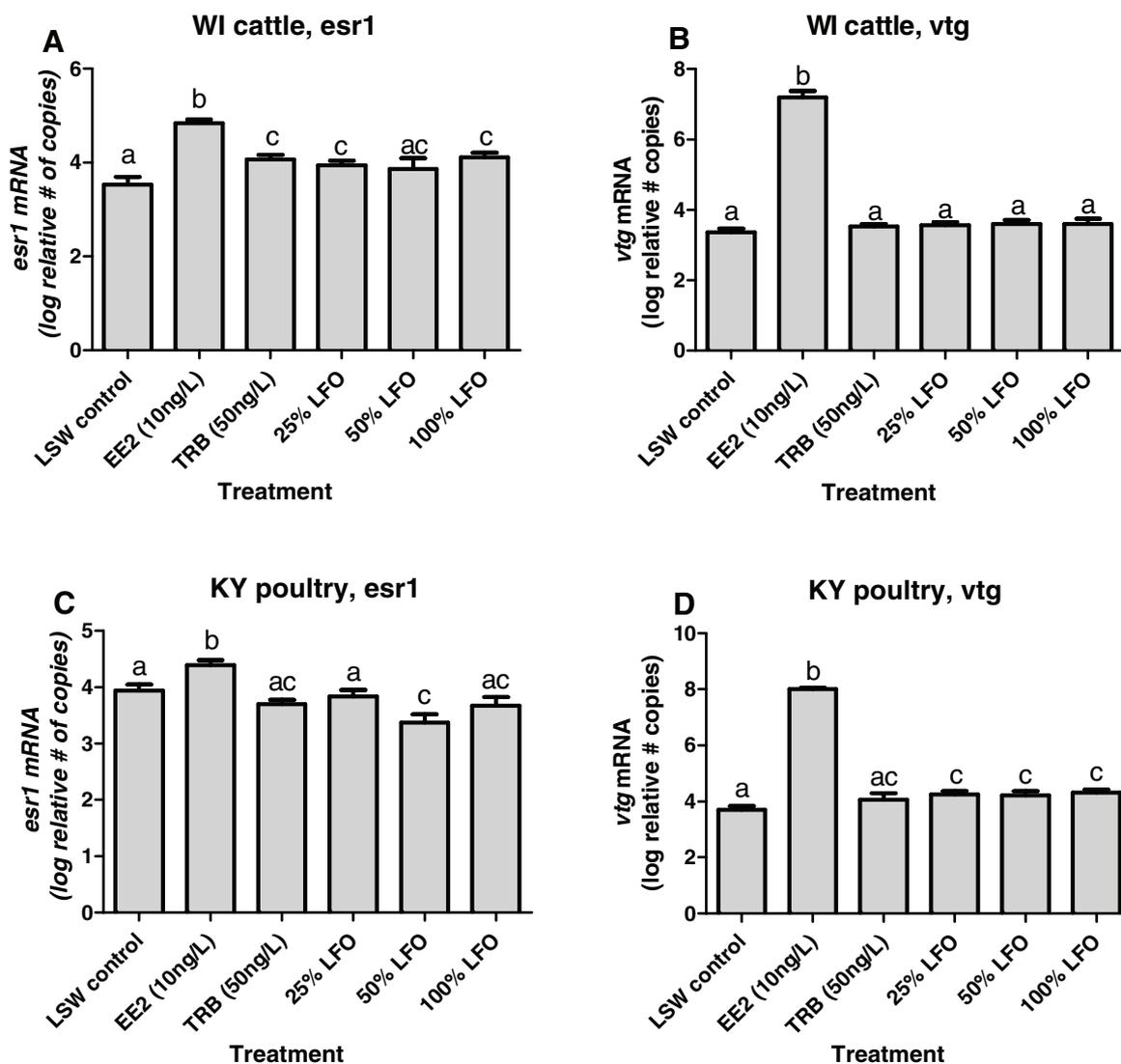


Figure 3. Relative abundance of mRNA transcripts measured in liver tissues from male fathead minnows exposed to dilutions of livestock farming operation (LFO) basin surface water (25%, 50%, and 100% LFO) diluted with control Lake Superior water (LSW), 10 ng/L 17 α -ethynylestradiol (EE2), or 50 ng/L 17 β -trenbolone (TRB) positive control: (A) Wisconsin (WI) cattle, estrogen-receptor α (*esr1*) mRNA; (B) WI cattle, vitellogenin (*vtg*) mRNA; (C) Kentucky (KY) poultry, *esr1* mRNA; and (D) KY poultry, *vtg* mRNA. Bars on graph represent mean (\pm standard error; $n = 7-8$) expressed as log relative number of copies. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

exposures. Currently, a specific molecular biomarker of androgen exposure has yet to be established [32]. However, previous examinations of estrogenic activity have repeatedly demonstrated that male fish exposed to estrogens can exhibit increased hepatic transcript abundance of *esr1* and *vtg* mRNA, as well as plasma VTG protein, all of which normally are present at undetectable or low levels in males [21,22,33-35]. Using relative *esr1* and *vtg* transcript abundance as indicators of estrogen exposure in males, results from the present in vivo fathead minnow exposures are inconsistent with the analytical chemistry and in vitro bioassay results from the present study. Given the estrogenic response observed in the T47D-Kbluc assays and analytical detection of 3 endogenous estrogens at 3 of the sites, we might have expected to see a biological response (i.e., upregulation of *esr1* and/or *vtg*) in the corresponding exposed male fish. However, *esr1* and *vtg* transcript abundances were not consistently upregulated in fish exposed to water from the sites exhibiting the highest estrogenic response in vitro. In addition, plasma VTG protein concentrations were not affected in males exposed to runoff water from the New York dairy site

(the sample that elicited the highest estrogenic response in the T47D-Kbluc assays). One explanation for the lack of biological response in the fish may be that the 48-h duration of the in vivo exposure was insufficient. For example, a previous study conducted by Ekman et al. [36] demonstrated that VTG protein can be significantly induced in male fathead minnows as early as 24 h after initiation of exposure to 100 ng/L of EE2, but that 4 d of exposure is required to induce detectable plasma VTG at a lower concentration of 10 ng/L of EE2 [36]. Because EE2 equivalents estimated from the T47D-Kbluc assays in the present study only ranged from 0.09 ng/L to 3.30 ng/L, there may have been insufficient intrinsic "signal" to produce a response in the fish in only 48 h. An alternative explanation for the seeming lack of congruence between the in vitro and in vivo data could be that the estrogenic activity measured in vitro is contributed by estrogenic compounds that are unavailable to or readily metabolized by the fish. Additionally, the potential for biodegradation of hormones needs to be considered in the comparison of analytical chemistry measurements and the lack of in vivo responses. Although the water samples for analytical

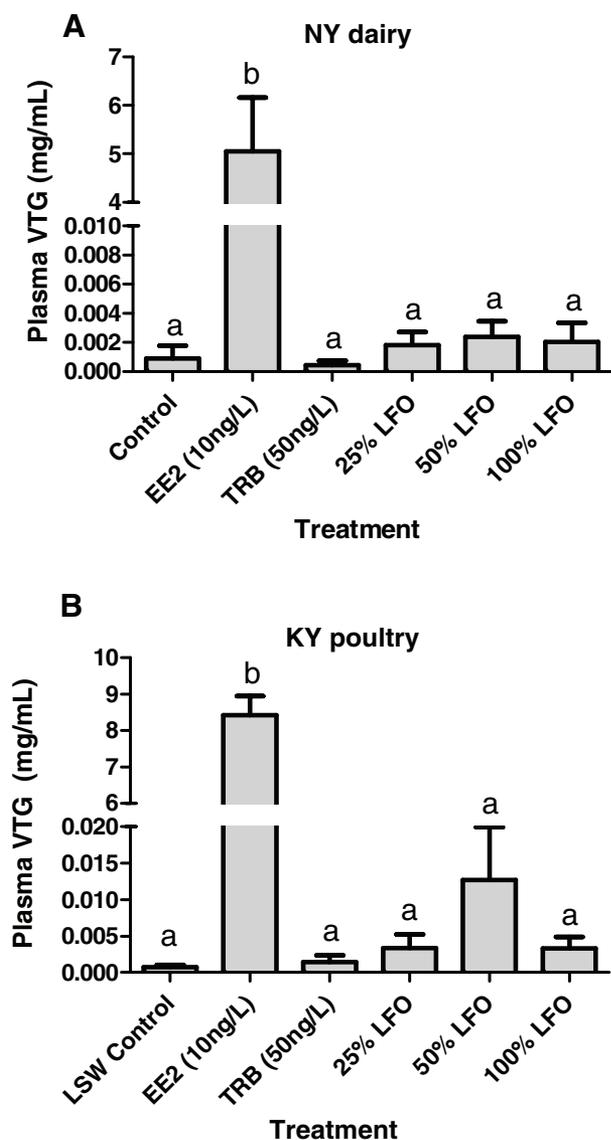


Figure 4. Plasma vitellogenin (VTG) protein concentrations from male fathead minnows exposed to dilutions of livestock farming operation (LFO) basin surface water (25%, 50%, and 100% LFO) diluted with control Lake Superior water (LSW), 10 ng/L 17 α -ethynylestradiol (EE2), or 50 ng/L 17 β -trenbolone (TRB) positive controls in 2 studies with surface water from (A) the New York (NY) dairy basin and (B) the Kentucky (KY) poultry basin. Bars on graph represent mean (\pm standard error; $n=8$). Different letters indicate statistically significant differences between treatments ($p < 0.05$).

chemistry and in vivo exposures were collected concurrently in the field, there is potential for chemical degradation prior to and during the in vivo exposures. In any instance, a logical conclusion from the in vivo portion of the study might be that, although estrogenic chemicals were detectable analytically and the samples were estrogenic in vitro, livestock farming operation runoff was not potent enough to elicit an estrogenic response by the fish over a short-term exposure.

In conclusion, the results from the present study highlight the value of utilizing a combination of techniques to obtain a more comprehensive representation of an environmental chemical mixture. Analytical chemistry, in vitro bioassays, and in vivo testing each have their own utility, and when the strengths of these targeted and nontargeted analyses are integrated into a single study, more nuanced conclusions can be drawn. For example, in the present study, analytical chemistry results alone

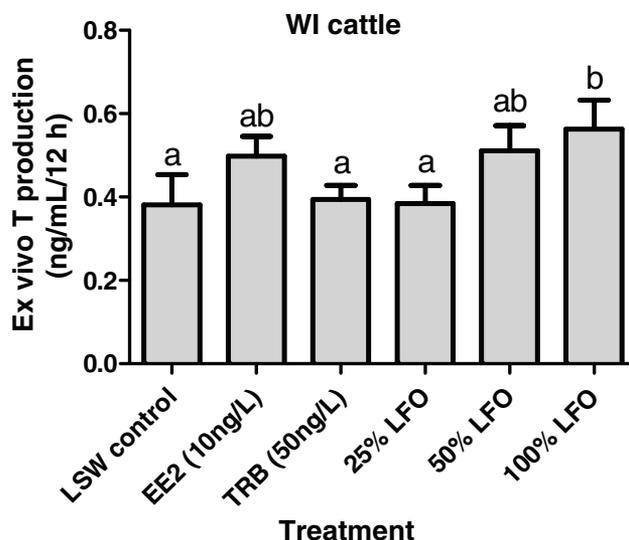


Figure 5. Effects of a 48-h static-renewal exposure to dilutions of livestock farming operation (LFO) basin surface water (25%, 50%, and 100%) diluted with control Lake Superior water (LSW), 10 ng/L 17 α -ethynylestradiol (EE2), or 50 ng/L 17 β -trenbolone (TRB) positive controls on male fathead minnow gonadal ex vivo testosterone (T) production. Bars on graph represent mean (\pm standard error; $n=8$). Different letters indicate statistically significant differences between treatments ($p < 0.05$).

could suggest that only 3 of the 6 sites contained targeted estrogenic compounds. By employing in vitro assays, estrogenic activity was detected in all 6 sites, which was not readily inferred from the analytical results. However, the in vivo results further reveal that although the water was estrogenic, the overall potency and/or composition was not sufficient to produce a significant in vivo response over a relatively short duration of exposure, such as may be experienced in situ as a result of a runoff event.

SUPPLEMENTAL DATA

Tables S1.

Figures S1–S3. (580 KB DOCX).

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Disclaimer—This article has been reviewed in accordance with official USEPA and USGS policy. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US government. Conclusions drawn in the present study neither constitute nor reflect the view or policies of the USEPA.

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