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Bioassay of estrogenicity and chemical analyses of estrogens in streams across the United States associated with livestock operations

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ABSTRACT

Animal manures, used as a nitrogen source for crop production, are often associated with negative impacts on nutrient levels in surface water. The concentrations of estrogens in streams from these manures also are of concern due to potential endocrine disruption in aquatic species. Streams associated with livestock operations were sampled by discrete samples ($n = 38$) or by time-integrated polar organic chemical integrative samplers (POCIS, $n = 19$). Samples were analyzed for estrogens by gas chromatography-tandem mass spectrometry (GC-MS^2) and estrogenic activity was assessed by three bioassays: Yeast Estrogen Screen (YES), T47D-KBluc Assay, MCF-7 Estrogenicity Screen (E-Screen). Samples were collected from 19 streams within small (~1–30 km^2) watersheds in 12 U.S. states representing a range of hydrogeologic conditions, dominated by: dairy (3), grazing beef (3), feedlot cattle (1); swine (5); poultry (3); and 4 areas where no livestock were raised or manure was applied. Water samples were consistently below the United Kingdom proposed Lowest Observable Effect Concentration for 17β-estradiol in fish (10 ng/L) in all watersheds, regardless of land use. Estrogenic activity was often higher in samples during runoff conditions following a period of manure application. Estrone was the most commonly detected estrogen (13 of 38 water samples, mean 1.9, maximum 8.3 ng/L). Because of the T47D-KBluc assay's sensitivity towards estrone (1.4 times 17β-estradiol) it

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was the most sensitive method for detecting estrogens, followed by the E-Screen, GC-MS^2, and YES. POCIS resulted in more frequent detections of estrogens than discrete water samples across all sites, even when applying the less-sensitive YES bioassay to the POCIS extracts.

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1. Introduction

Livestock operations across the U.S. generate more than 1.1 billion tons of manure annually (USDA, 2009), much of which is land-applied as fertilizer. In some instances, however, such land applications of animal manure can contribute to degradation of surface water quality. Historically, concerns pertaining to animal manure have dealt primarily with nutrient issues (Jongbloed and Lenis, 1998). More recently, concerns have arisen about other constituents present in manure, such as naturally occurring hormones (Hanselman et al., 2003).

Only limited information has been published regarding the fate of estrogenic compounds originating from animal feedlot/feeding operations (AFOs) that might be transported into receiving waters (Leet et al., 2011). Therefore, the U.S. Geological Survey (USGS) led a multi-year study to assess the occurrence of a broad range of chemical and microbial contaminants derived from AFOs in streams across the United States. Study sites included small, headwater watersheds (~1–30 km^2) chosen to be representative of the major livestock types (swine, poultry, beef, and dairy) located across the nation over a range of geographic and hydrogeologic settings. A similar set of "rural, background" watersheds that had similar land use (e.g. cropland), but with no livestock operations or manure applications, also were sampled to establish baseline conditions. Samples consisted of manure, discrete and time-integrated water samples (using passive sampling devices), and streambed sediment. All samples were analyzed for a comprehensive set of chemical and microbial constituents, including a number of chemicals that are known to be estrogenic. In this paper, we present data only on estrogens and estrogenic activity in discrete and time-integrated water samples collected.

Of particular interest are estrogenic chemicals that have been linked to endocrine disruption in fish (Metcalfe et al., 2001; Nash et al., 2004; Gross-Sorokin et al., 2006; Routledge et al., 1998). Long-term exposure to these estrogens can have population-wide implications for native fish (Kidd et al., 2007). The Environment Agency (United Kingdom) concluded "the weight of evidence for endocrine disruption in fish is sufficient to develop a risk management strategy for estrogenically active effluent that discharge to the aquatic environments" (Gross-Sorokin et al., 2006).

A number of *in vitro* assays have been developed to determine the "total" estrogenic activity of samples relative to 17β-estradiol (E2), which, to date, is the most potent natural estrogen *in vivo*. Data from these assays are reported as estradiol equivalents (E2Eq). Because of the large number of chemicals that are potentially estrogenic and the high cost associated with identifying these chemicals using analytical technologies of adequate sensitivity (including mass spectrometric methods) *in vitro* bioassays have become an attractive alternative to chemical analysis. As a component of this study, estrogenic activity was compared using three of these assays, the Yeast Estrogen Screen (YES), T47D-KBluc Estrogen Assay (T47D-KBluc), and MCF-7 BOS Estrogenicity Screen (E-Screen). There have been previous comparisons of estrogen bioassays with pure chemicals (Soto et al., 1995; Li et al., 2004; literature review comparison by Fang et al., 2000) and with environmental samples (Kusk et al., 2011; Leusch et al., 2010). However, our study is unique in providing concomitant discrete and time-integrated polar organic chemical integrative sampler (POCIS) water samples from agricultural watersheds and comparing assay results to chemical analysis of these samples.

The three bioassays used differ in cell source and biological endpoint, as well as in practical aspects such as time, expense, and equipment required. The YES and T47D-KBluc screen are transcriptional activation assays. The YES uses yeast cells transfected with the human estrogen receptor, while T47D cells, like MCF-7 BOS cells, are human breast cancer cell lines, and both naturally express the α- and β-estrogen receptors (ER). The T47D cells were stably transfected with triplet estrogen-responsive element-promoter-luciferase reporter gene construct. The yeast cells in this study have been transfected with expression plasmids carrying a reporter gene (*lac*-z) situated downstream from a promoter sequence, which incorporates an estrogen response element (ERE). In contrast to the transcriptional activation assays, the E-Screen endpoint measures cellular proliferation. In addition to a contemporary evaluation of the estrogenicity of surface waters associated with livestock operations, the objectives were to compare the usefulness of the selected bioassays to chemical analyses and to determine estrogen concentrations in discrete water samples versus those collected by POCIS.

2. Materials and methods

2.1. Site descriptions

Surface waters from 19 headwater basins, in 12 states, were sampled. Each basin was chosen to reflect a homogeneity of agricultural land usage, including the major livestock types: dairy (3), grazing beef (3), feedlot cattle (1), swine (5), poultry (3, broilers), and rural background (4) where no livestock were raised or manure was applied (Table 1). Locations were chosen in an attempt to study contaminant inputs from specific animal types in different hydrogeologic settings, with no or minimal input from other livestock animal types. The objective was to obtain water samples under two different flow conditions (e.g., baseflow and runoff). For basins with specific periods of manure application, the baseflow (BF) sample was collected prior to application and the runoff (RO) sample was

Table 1 – Site descriptions.

Source of livestock manure	State	Drainage area and basin land use	Livestock numbers and percent of land receiving manure	Landscape and major soils	Range of measured flow rates at sampling (m^3/s)	Time of manure application relative to sampling
Control–(only chemical fertilizer usedon fields)	PA	1.3 km^2 60% forested, 40% row crops 10 septic systems Located above swine basin	None	Hills – Well drained stony loam and silt loam soils formed in sandstone and shale residuum, with medium runoff potential.	0.011–0.034	
	OH	18.1 km^2 Row crops Heavily tile drained	None	Flat Glacial Lake Plain – Poorly to very poorly drained loam and clay loam soils with low runoff potential.	0.006–0.283	
	IN	6.2 km^2 Row crops Tile drains present	None	Loess-Covered Flat Glacial Till Plain – Poorly to somewhat poorly drained silt loam and silty clay loam soils with medium runoff potential.	0.020–0.351	
	IA	2.1 km^2 Most of basin in corn and soybeans	None	Deep Loess over Undulating Glacial Till– Well drained silt loam and silty clay loam soils on moderate slopes, prone to runoff.	0.016–0.017	
Poultry	MD	2.6 km^2 Corn, soybeans, and small grains ~3 septic systems	Confinement rearing of 480,000 broilers/yr. Manure stored in sheds. 80% of land receives poultry litter.	Flat Coastal Plain – Very poorly drained mucky loams and well drained sandy loams with low runoff potential.	0.011–0.017 Intermittent stream	Baseflow collected prior to application of litter, runoff afterwards.
	KY	5.7 km^2 404hectares of corn 4 septic systems	Confinement rearing 470,000 broilers/yr Manure stored in sheds.	Loess-Covered Hills – Well drained silt loam soils on steep slopes, prone to runoff.	0.0002–0.001	*No manure applied this year, too wet.*
	AR	6.7 km^2	Poultry (~ 100 head of grazing cattle also present).	Hills – Well drained stony sandy loam and silt loam soils formed in sandstone and shale residuum, prone to runoff.	0.003–0.270	*Litter was primarily applied outside of basin.*
Swine	PA	0.8 km^2 23% forested, 72% ag 5% grazing pasture 5 septic systems	70% of land typically receives swine manure (40head of grazing cattle, but no access to stream).	Hills – Well drained stony loam and silt loam soils formed in sandstone and shale residuum with medium runoff potential.	0.006	*No manure application during study period.* Only baseflow sample only collected.
	IA-1	~9.8 km^2 95% row crops Heavily tile drained	~ 9000 hogs ~75% of land receives manure post-pit/ lagoon storage.	Flat Glacial Till Plain – Well to poorly drained loam to silty clay loam soils with medium runoff potential.	0.028–1.165	Same IA stream as IA-2 (see description below).
	IA-2	33.7 km^2 90% row crops Heavily tile drained	~ 25,000 hogs 75% of land receives swine manure post-pit/lagoon storage.	Flat Glacial Till Plain – Well to poorly drained loam to silty clay loam soils with medium runoff potential	0.009–0.096–2.55 [a]E	Baseflow collected prior to manure application, but wet conditions delayed manure application into winter, with two samples taken post-application, (winter baseflow and spring runoff during snowmelt).

(*continued on next page*)

Table 1 – (*continued*)

Source of livestock manure	State	Drainage area and basin land use	Livestock numbers and percent of land receiving manure	Landscape and major soils	Range of measured flow rates at sampling (m^3/s)	Time of manure application relative to sampling
	IN	26.9 km^2 82 % row crops 7% developed 6% pasture 5% forest Tile drains present	~15,000 hogs ~ 50% of land receives swine manure post pit storage.	Loess-Covered Flat Glacial Till Plain – Well to poorly drained silt loam and silty clay loam soils with medium runoff potential.	0.0002–0.60	Baseflow collected prior to manure application, runoff after application.
	NC	<2.6 km^2	1500 hogs Spray application of effluent from 2nd treatment lagoon.	Piedmont Uplands – Well drained sandy loam soils formed in igneous residuum prone to runoff.	0.006–0.006	Multiple spray applications of swine manure to fields. Drought conditions, so no runoff collection.
Beef (Grazing)	VA	5.2 km^2 47% forested 53% pasture	Cow/calf operation 1000 breeding cows w calves 53% of land receives manure from grazing cattle.	Piedmont Plateau – Well drained, stony loam soils on steep slopes with medium runoff potential.	0.008–0.008 Perennial stream, 2" to deeper pools	Cattle had stream access immediately above sampling sites during baseflow, but moved to a different portion of the basin during runoff sampling.
	WI	4.1 km^2	Cow/calf operation 33 cows breeding cows with calves, ~3 steer, with stream access.	Sedimentary Bedrock – Shallow, well drained silt loam soils on steep slopes prone to runoff	0.037–0.076	Cattle had stream access during baseflow and runoff.
	IN	23.1 km^2 81% row crops, 7% developed 6% pasture 5% forest	20 head cattle with stream access.	Loess-Covered Flat Glacial Till Plain – Well to poorly drained silt loam and silty clay loam soils with medium runoff potential.	0.0006–0.634	Cattle had stream access during baseflow and runoff.
Cattle (Feedlot)	IA	13.2 km^2 ~ 90% Row crop	2000 head cattle Manure application: 50% fall, 20% winter, 30% spring.	Deep Loess over Undulating Glacial Till – Well drained silt loam and silty clay loam soils on moderate slopes prone to runoff.	0.122–0.153	Baseflow collected prior to application of manure, runoff afterwards (1st event, though small discharge).
Dairy	MI	15.0 km^2 67% ag 16% forest 9% wetland 6% urban 2% other	~ 2000 confined cows 60% of land receives dairy manure post-pit/lagoon storage.	Flat Glacial Till Plain – Well to poorly drained loam and clay loam soils with medium runoff potential.	0.057–0.105	Baseflow collected prior to application of manure, runoff afterwards.
	WI	31.1 km^2	500 confined cows Land application of dairy manure.	Loess-Covered Sedimentary Bedrock – Shallow, well drained silt loam soils on steep slopes prone to runoff .	0.309–1.557	Runoff collected after manure application, baseflow collected ~4 months later.
	NY	26.7 km^2 79% wheat & corn Some tile drains	3000 pastured cows No stream access. ~ 70% of land receives dairy manure	Undulating Glacial Till – Deep, well-drained, silt loam soils on moderate slopes with medium runoff potential.	0.088–0.850	Baseflow collected prior to application of manure, runoff afterwards.

[a] E, estimated flow as conditions not amenable for measurement.

collected after application. In several locations this was not possible for various reasons, including dry conditions, where runoff failed to occur, and when water-saturated soils prevented application of manure (each state defines acceptable conditions for field application of animal manure). An unexpected release of swine manure from a storage lagoon occurred upstream of one sampling location, and an unplanned water sample was collected at this time, in response to this release.

2.2. Sample collection

2.2.1. Water

Unfiltered stream water samples (6 L) for bioassay analyses were collected into 1-L amber, baked-glass bottles, and 500 mL were collected into high density polyethylene bottles for chemical analysis. All water samples were collected from the centroid of flow and immediately chilled and shipped overnight to the participating laboratories. Field blanks ($n = 11$), prepared using nanopure water (npH_2O), and field replicates ($n = 2$) were collected under the identical conditions. Other parameters measured at the time of sampling included stream flow, dissolved oxygen, pH, specific conductance, and water temperature (Supporting information, Table S1).

2.2.2. POCIS

Polar organic chemical integrative samplers (POCIS) were deployed at all sites as a basic screening tool to determine hormones that may be present in the streams episodically, or that were transported in concentrations too low to be detected by conventional sampling techniques (Alvarez et al., 2004). These samplers were used to provide a time-integrated measure of dissolved estrogens in the streams at each study basin. The POCIS, consisting of a solid-phase sorbent enclosed between two polyethersulfone membranes, is designed to sample organic chemicals with high to moderate water solubilities (Alvarez et al., 2004). Protective stainless steel deployment canisters containing eight POCIS (200 mg Oasis HLB sorbent, Waters Corp., Milford, MA: 41 cm^2 sampling surface area) were placed at each study site for an average of 44 days to include the period of manure application. Details on the individual site conditions are given in Table 1. Due to limited water depth at a few sites, deeper streambed recesses were created to ensure the POCIS remained submerged throughout the deployment period. Either small pools were dug out to collect water, or partial dams were constructed to hold back water. Such alterations could impact stream flow, and thus the results. At all sites, POCIS field blanks consisted of extracts of POCIS samplers that were exposed to on-site air during the times of POCIS field-sample deployment and retrieval. Upon retrieval, the POCIS were shipped in air-tight containers on ice to the laboratory where they were stored at −20 °C until processed.

2.3. Sample preparation

2.3.1. Water samples for bioassays

Water samples were filtered upon arrival at the laboratory (25-mm diameter, 0.7-μm nominal pore size glass-fiber filter, Thermo Fisher Scientific, Waltham, MA). One liter aliquots (5 replicates) of the sample were extracted on Oasis HLB solid-phase extraction columns (SPE: 200 mg, 5 cc). Columns were conditioned prior to sample extraction with 5 mL of methanol (MeOH) followed by 4 mL of npH_2O. Each 1 L replicate was weighed before extraction to obtain the exact volume of extracted sample (using a density of 1 g/mL). Following extraction, columns were rinsed with 1 mL npH_2O and dried under vacuum (20 min). Columns were eluted with 2 × 4-mL of HPLC grade MeOH into 10 mL glass centrifuge tubes, and eluents were concentrated to ~0.5–1 mL using N_2 and a 45 °C water bath. The 5 replicate sample extracts (tubes) were then combined into one silanized tube. Post-transfer MeOH rinses of each tube were added to the final combined extract, which was then concentrated to ~100 μL. Prior to a solvent exchange (required for bioassays), 10 μL of npH_2O was added to each sample, followed by nitrogen evaporation (as described above) of the extract to 10 μL. The composite sample extract was brought to a final volume of 500 μL with ethanol (EtOH). One 100-μl aliquot of the composite extract was shipped on ice overnight to each of three partnering laboratories for bioassay, and two aliquots were archived at −10 °C. To estimate extraction recovery, five 1 L replicates of npH_2O in amber glass bottles were spiked with either 30 ng/L of 17β-E2 or 17a-ethinyl estradiol (EE2), mixed and immediately extracted and processed as described above. Spiked-extracts were further processed and analyzed by gas chromatography-tandem mass spectrometry (GC-MS2) using procedures described in Section 2.3.3 for the POCIS extracts (recovery results in Table 2).

2.3.2. POCIS samples

Individual POCIS were extracted with MeOH and processed according to previously described methods (Alvarez et al., 2008; Alvarez, 2010), providing one extract from one POCIS for each of the three assays. Chemical analysis for hormones was performed on a composite of two POCIS extracts prepared as described in Section 2.3.3. Extracts were concentrated by rotary and N_2 evaporation. Extracts were transferred to amber ampules and resuspended in 0.5 or 1.0 mL of EtOH (bioassays) or MeOH ($GC\text{-}MS^2$), sealed, and shipped to partnering laboratories for estrogenic screening and chemical analysis.

Table 2 – Extraction and bioassay performance on spiked samples.

Matrix and fortification	Estrogen (ng/L ± SD)			$GC\text{-}MS^2$
	E-Screen	T47D-KBluc	YES	ng/L[b]
npH_2O E2 30 ng/L	22 ± 3.0[a]	19 ± 2.1[a]	17 ± 0.3[a]	18.8
npH_2O EE2 30 ng/L	31 ± 2.7[c]	25 ± 7.7[c]	34 ± 0.5[d]	15.5

a Estradiol equivalents (E2Eq) are based on a 17β-E2 curve run concurrently with the extracted samples.
b $GC\text{-}MS^2$ analyses of extracts prepared for bioassay analyses. These values are corrected by isotope-dilution quantification based on isotope addition to the extract, and therefore are not corrected for losses that may have occurred in extraction.
c EE2 values are based on EE2 curve run concurrently with the bioassay.
d EE2 values are corrected for bioassay's relative estrogen potency factor for EE2.

2.3.3. Chemical analysis of water and POCIS samples

Estrone (E1), 17α- and 17β-E2, estriol (E3), and 17α-ethinyl estradiol (EE2) were determined in unfiltered water samples as previously detailed (Foreman et al., 2012). Deuterium- or ^{13}C-labeled isotope-dilution standard (IDS) analogs of the estrogens were added to the water sample (100 μL of 0.5-ng/μL IDS compounds to 0.5 L sample) which was passed through an C18 SPE disk. Following extract cleanup using Florisil SPE, method compounds were converted to trimethylsilyl derivatives and analyzed by GC-MS^2 (model Quattro-micro-GC, Waters Corp., Milford, MA) operated in electron-impact ionization mode. Injection volume was 2 μL of a 200-μL final extract. At least three precursor-to-product ion transitions were monitored per analyte. Positive analyte identification required the presence of at least two unique product ions, with ion ratios not deviating from those in a standard by more than the specified tolerances (Foreman et al., 2012). Analyte concentrations were determined using isotope-dilution quantification and standard curves. Detection levels were 0.4 ng/L for all estrogens except E3 (1 ng/L) with reporting levels (quantitation) twice the detection level (Foreman et al., 2012). Recoveries of the IDS in samples were used to evaluate sample-specific method performance. Laboratory reagent-water blank and spike samples were analyzed with each set of environmental samples. Laboratory spike samples (positive controls) were fortified with 25 ng/L of the estrogens in 0.5 L (in addition to the spiked samples used to estimate extraction performance of samples used for bioassay evaluation).

The POCIS MeOH extracts were fortified with the same IDS compounds used for the water samples, and concentrated to dryness using N_2. Following reconstitution in 5% MeOH in dichloromethane, the extracts were further processed using Florisil SPE, and the method compounds were derivatized and analyzed by GC-MS^2 using the same procedures as applied to the water samples. Lab blank and spike samples were prepared using MeOH to simulate the POCIS MeOH extracts. Analyte concentrations in POCIS samples were expressed as ng/POCIS.

2.4. YES assay

Yeast cells transfected with the human estrogen receptor (hERα), initiate a cascade of events upon binding with an estrogenic agonist that result in the release of β-galactosidase into the growth media, cleaving galactose from the chromogenic substrate (chlorophenol red-β-D-galactopyranoside – CPRG), with a resultant color change measured at an absorbance (A) of 540_{nm}, (Routledge and Sumpter, 1996). Quantitation of the sample's estrogenic activity is based on interpolation from yeast grown in 17β-E2 (Sigma Chemicals, St. Louis, MO; 4×10^{-12} to 1×10^{-8} M). The 96-well test plates were treated as follows: first row first column, 17β-E2 standard (1×10^{-8} M); first column subsequent rows – alternating replicates of EtOH (200 μL negative controls) and test sample (25 μL of extract diluted with 175 μL EtOH for the water samples and 50 μL of extract diluted with 150 μL EtOH for the POCIS) to provide triplicate analysis of the test sample. After adding EtOH (100 μL) to all wells in columns 2–12, column 1 wells were serially diluted 1:1 across the remaining 11 columns of the plate. The liquid (sample and/or solvent) in each well was allowed to evaporate prior to adding assay medium (200 μL containing ~ 4×10^7 recombinant yeast cells and CPRG). The plates were gently agitated, sealed, and incubated at 30 °C for up to 72 h. Plates were monitored daily to assess progress of the CPRG conversion by yeast cells + 17β-E2. At 72 h, the A 540_{nm} (free chlorophenol red released from CPRG by galactosidase) was read on a plate reader, and adjusted for cell turbidity by subtracting A 620_{nm} cells + sample – A 620_{nm} cells + EtOH (Rastall et al., 2004). No measure of toxicity originating from the extracts was performed other than visual observations of cell death. Detection limits were 0.042 E2Eq ng/L in water samples and 0.085 ng/POCIS in original samples, quantitation limits were twice the LODs. Concentrations were calculated using sigmoidal dose response non-linear regression (GraphPad Prism Version 5, La Jolla, CA). While cells are exposed to 17β-E2 concentrations over almost 4 orders of magnitude, calculated concentrations typically fell over less than two orders of magnitude on plate. The ½ maximal effective concentrations (EC_{50s}) of other estrogens (E1, 17α-E2, E3, and EE2) were the respective concentrations at which ½ the maximum 17β-E2 response (the expression of the *lac-z* reporter gene following binding of E2 to the estrogen response element) was obtained. Relative estrogen potencies (REPs) or E2Eq of other estrogens were calculated by dividing the EC_{50} of 17β-E2 by the EC_{50} of the other estrogen. Data from GC-MS^2 analysis of the samples were compared to bioassay data by calculating the sum of predicted E2Eqs. GC-MS^2 concentrations for each estrogen were multiplied by their respective REPs for the YES assay and summed. That is: predicted E2Eq = $\sum$(ng/L E1 * REP $E1_{YES}$) + (ng/L 17α-E2 * REP 17α-$E2_{YES}$) + (ng/L E3 * REP $E3_{YES}$) + (ng/L EE2 * REP $EE2_{YES}$) = $E2Eq_{cYES}$.

2.5. T47D-KBluc assay

T47D-KBluc cells (ATCC # CRL-2865) which contain endogenous ER and stably express a triplet estrogen response element-luciferase promoter–reporter gene construct were maintained and assayed as described by Wilson et al. (2004). Briefly, one week prior to testing samples, media containing 10% fetal bovine serum (FBS) was replaced with media containing 10% charcoal dextran stripped FBS (CD-FBS) to remove estrogens from the cultures. Mid-week, cells were fed with fresh CD-FBS media. At the end of the week (8th day), cells were harvested from flasks and plated in 96-well plates (1×10^4 cells/well in 100 μL CD-FBS; Costar 3610, Corning Inc., Corning, NY). The following day, cells were treated with extracts serially diluted in 5% CD-FBS media, with <0.3% final EtOH concentration. Typically the initial extract dilution was 1:1000 into media followed by 2-fold serial dilutions in media. A standard curve of 17β-E2 from 3×10^{-14} to 1×10^{-10} M for extracts of water samples and 3×10^{-13} to 3×10^{-11} M for extracts from POCIS was run concomitantly on each plate. Each sample was tested in a minimum of two separate experiments with 4 replicate wells per experiment. Luciferase activity was determined after a 24 h incubation on a luminometer (BMG Lumistar, BMG Lacteck, Durham, NC) and quantified in relative light units (RLU). For data analysis, RLU per well were normalized to vehicle control (fold over vehicle) and then log transformed to correct for heterogeneity of variance (log fold). Log fold value of each well was then converted to percent of maximal 17β-E2 response (using 17β-E2 curve on same plate) before replicate plate data were

combined. Curve fits were prepared using a non-linear fit of the combined data (log(agonist) versus response – variable slope; GraphPad Prism v5.0). The dilution of sample extract that produced 50% of the maximal E2 response was used to calculate E2Eq (in ng/L) adjusting for the appropriate concentration factors of original water (or POCIS) volume. Detection limit was 0.005 ng/L E2Eq, and quantitation limit was 0.01 ng/L in water samples. Quantitation limit of E2Eq from POCIS in original samples was 0.08 ng/POCIS. The ½ maximal effective concentrations (EC_{50s}) of other estrogens (E1, 17α-E2, E3, and EE2) were the respective concentrations at which ½ the maximum 17β-E2 response was determined. The REPs and chemically predicted E2EQs ($E2Eq_{cKBluc}$) were calculated as described in Section 2.4. Specificity of estrogenicity in positive samples was confirmed by estrogen receptor antagonist ICI 182,780 (Tocris, Ellisville, MO) as described by Rassmussen and Nielsen (2002).

2.6. E-Screen assay

E-Screen was performed essentially as previously reported using MCF-7 BOS cells (Shappell, 2006). Cellular proliferation in the presence of sample extract was compared to proliferation in the presence of 17β-E2. Water sample extracts were diluted in steroid-free medium (with no phenol red and 10% CD-FBS) and tested on 5 wells of cells, in at least two experiments. In the 6th well, toxicity was assessed by comparing cellular proliferation in the presence of sample plus 17β-E2. A linear regression of the standard curve (over the linear range of the standard curve of the log concentration versus absorbance, Microsoft Office, Excel 2003, Redmond, WA) was used to calculate sample E2Eqs. Most samples fell within the linear range of the 17β-E2 curve (1×10^{-12} to 1×10^{-11} M) when tested at 4–8 times the original concentration. The exceptions were the water blank samples spiked with 30 ng/L EE2 or 17β-E2 and the unplanned sample collected downstream of a storage lagoon release, that were diluted to 0.04 times the original concentrations for the assay. Quantitation limits were ~0.03 ng/L of 17β-E2Eq and ~0.1 ng/POCIS in original samples.

POCIS extracts were treated the same way as water samples, with an original 500 μL volume (equivalent to an extract from one SPE) diluted 200–1600 times to obtain cellular responses in the linear range of the assay. Evidence of toxicity was determined by evaluating cellular proliferation in the presence of samples spiked with 17β-E2 versus 17β-E2 alone. Specificity of estrogenicity in positive samples was confirmed as described for the T47D-KBluc-KBLUC assay (Section 2.5). The EC_{50s} of the other estrogens were established by determining the concentration that ½ the maximum proliferative response to 17β-E2 was obtained. REPs and chemically predicted E2Eqs ($E2Eq_{cE\text{-}Screen}$) were calculated as described in Section 2.4.

3. Results and discussion

3.1. 17β-E2 equivalents by bioassay

The REP, or rankings, and EC_{50}'s of the various estrogens were bioassay specific (Table 3). The need to report these values for each laboratory is evident when comparing, for instance, the various relative potencies by YES. Estrone, the most commonly detected natural estrogen in surface waters associated with livestock operations or fields where animal manure was applied as fertilizer, has been reported to have REPs of 0.68, 0.96 and 0.02 (Thorpe et al., 2006; Coldham et al., 1997; Cespedes et al., 2004, respectively). Disparate REP values (difference of 10–50 times) have also been reported for E3 and EE2 (Coldham et al., 1997; Cespedes et al., 2004). While the T47D-KBluc assay was first described in 2004 (Wilson et al.), multiple published comparisons of REPs are not currently available. In contrast, there have been multiple reports of REPs for E-Screen, with essential agreement among them for the various estrogens (Soto et al., 1995; Gadd et al., 2010; a compilation in Leusch, 2008; authors' data presented in Table 3).

Literature reports comparing relative estrogenic activity of environmental samples using two different bioassays sometimes have included chemical analysis. Fang et al. (2000) compared estrogen receptor competitive binding assays (ER binding assays), yeast-based reporter gene assays, and MCF-7 cell proliferation assays (E-Screen) by examining data from five laboratories for more than 29 chemicals. While laboratory variability explained some variability of results, the differences in assay response to different types of compounds (e.g. antiestrogens, phytoestrogens, alkylphenols) also were evident.

Assessment of an assay's sensitivity, reproducibility, ease and cost of analysis are the typical metrics considered when choosing an *in vitro* assay for estrogenicity. Specific consideration might not be given to the expected estrogenic contaminants present in the environmental samples collected and the assay's capacity to detect their occurrence. For example, in studies focusing on water samples impacted predominantly by animal inputs, it is important to know what natural estrogens persist, what their relative (*in vivo*) biological activity is, and the quantitative capacity of each assay for the estrogens of interest. While the most biologically potent synthetic estrogen may be EE2 in fish *in vivo* (Brian et al., 2005), the probability of its presence, even in effluent from wastewater treatment plants is often negligible (Allinson et al., 2010; Belfroid et al., 1999; Sellin et al., 2009). In a USGS study of U.S. streams in 27 states with a bias towards sampling downstream of intense urbanization or livestock production, EE2 was not detected in 68 of 72 samples (5 ng/L detection limits, Barnes et al., 2002 Table 6, Method 5).

The most salient point to be gleaned from the results in Table 3 is the T47D- KBluc assay's capacity to detect estrone. While 17β-E2 may be the most biologically active of the *natural* estrogens *in vivo*, it is typically found at very low concentrations in stream water. In the US stream study (Barnes et al., 2002) 17β-E2 was not detected in 55 of 62 samples (5 ng/L detection limit). Concentrations ranged from 1 to 93 ng/L for the 7 samples having quantifiable detections, and only 3 samples exceeded 10 ng/L (44, 73, and 93 ng/L). The current study supports this finding as 17β-E2 was not found above the quantitation limit (0.8 ng/L) by GC-MS^2 in any water sample with the exception of the unplanned sample collected as a result of an unexpected release of swine manure from a storage lagoon (Table 4). However, 17β-E2 was detected in 6 of 19 passive (POCIS) water samples (0.09–3.4 ng/POCIS unit per

Table 3 – Relative estrogen potency factors and (EC_{50s}) of various estrogens by *in vitro* bioassay and relative *in vivo* vitellogenin response in fish.

Assay[a]	Estrone	17α-Estradiol	17β-estradiol	Estriol	Ethinyl estradiol
E-Screen	0.012 (4.5×10^{-10} M)	0.017 (3.2×10^{-10} M)	1.0 (5.4×10^{-12} M)	0.15 (3.6×10^{-11} M)	1.0 (5.1×10^{-12} M)
T47D-KBluc[b]	1.4 (1.2×10^{-12} M)	0.007 (2.4×10^{-10} M)	1.0 (1.7×10^{-12} M)	0.23 (7.3×10^{-12} M)	7.23 (2.3×10^{-13} M)
YES	0.410 (2.2×10^{-9} M)	0.025 (3.8×10^{-8} M)	1.0 (4.6×10^{-10} M)	–[c]	0.47 (1.3×10^{-9} M)
Plasma vitellogenin in male fish	0.4 ± 0.23[d]	0.1[e]	1	0.1[d]	20 ± 6.9[d]

a Relative Estrogen Potency Factors are relative to 17β-E2 and ½ maximal effective concentrations (EC_{50s}), are from the laboratories that performed the analyses of the samples, not literature values from other labs.
b Previously published from Wilson laboratory in Bermudez et al. (2012).
c No response at 1×10^{-8} M estriol.
d Based on literature values for various species of fish, used by Caldwell et al. (2012) for predicted No Effect Concentration for the estrogens. Values represent mean ± SD for estrone (5) and ethinyl estradiol (6) literature values cited. *In vivo* data were not available on estriol, value estimated based on *in vitro* assay reports.
e Based on limited data for fathead minnows (Shappell et al., 2010) no other reports found.

30 day deployment, Table 5) documenting that either higher episodic or chronic low-level concentrations were present. In contrast, estrone was detected in 13 of 38 planned water samples and quantified in 13 of 19 passive water samples. More frequent detections of estrone than 17β-E2, were expected, because concentrations of estrone in manure treatment lagoons are typically 100 times those of 17β-E2 for most livestock types (Hutchins et al., 2007). Therefore, the screen with the greatest capacity to detect estrone, in this case T47D-KBluc, the signature chemical for animal manure contamination, has an advantage over assays that might more accurately reflect the cumulative estrogenic risk to fish.

The choice of the bioassay used for assessment of estrogenic activity should reflect the objective of the investigation. For example, if the objective is to detect any estrogenicity from animals, then the T47D-KBluc assay would be the choice, due to its high REP of estrone. If the objective is to obtain a measure of the total estrogenicity more in line with *in vivo* estrogenic activity, and therefore necessitating higher concentrations of estrone to be present before detection, the E-Screen could be more appropriate. While the T47D-KBluc assay might overestimate the estrogenic *in vivo* impact on fish, it would be less likely to "miss" identifying the presence of estrogens in water samples if estrone is the predominant estrogen present, compared to the E-Screen. The variability in literature reports of relative estrogen potencies for estrogens in the YES assay makes similar assay comparisons difficult. Other factors such as higher detection limits or assay ease of use (Section 3.2) might become the determining criteria for its use.

3.2. *Performance characteristics of assays*

Performance data for chemical analyses of the estrogens by GC-MS^2 generally were within acceptable ranges (Foreman et al., 2012). Mean method recoveries of the estrogens from laboratory spike npH_2O samples analyzed with field water samples ranged from 89 to 100%, with standard deviations (SDs) of 5–29% (data not shown). Mean isotope-dilution standard absolute recoveries for field water samples ranged from 79 to 87% with SDs of 10–22%, and were comparable to or better than in the npH_2O spikes (data not shown). For POCIS field samples analyzed by GC-MS^2, absolute recoveries of isotope-dilution standard fortified post-extraction ranged from 87 to 94% with SD's of 8–14% (data not shown).

A limited comparison of bioassay method performance was done by comparing chemical analyses of extracts of npH_2O spiked with 30 ng/L of 17β-E2 or EE2. Samples were prepared as described for the bioassay water procedure (2.3.1). Results of each bioassay are shown in Table 2. Also shown is the analyte concentration of the spiked sample extracts determined subsequently by GC-MS^2. The isotope-dilution standards used in the GC-MS^2 analyses were fortified post-extraction as deuterated compounds could not be added to samples prior to bioassay. Therefore, analyte losses that occurred during the bioassay sample preparation steps are not accounted for in the GC-MS^2 concentrations shown in Table 2. Results by the 3 bioassays are similar for 17βE2 (mean range 17–22 E2Eq ng/L), and compare well with the value determined by GC-MS^2 (19 ng/L). Corresponding recovery of 17β-E2 by these determinations range from 57 to 73%, indicating some loss or degradation during sample preparation as expected. Lower values for E2 in npH_2O have been observed in the E-Screen and attributed to "loss" of E2 to the container's walls in the absence of organic matrix. The same trend was found by chemical analysis reported by Holbech et al. (2006), where actual concentrations of estrogens (E1, 17β-E2, E3 in separate experiments) were found by LC-MS analysis to be ~50% of nominal in concentrations (mean of four separate experiments). Similarly, Routledge et al. (1998) found in fish exposure experiments, that the tank water concentrations of 17β-E2 as determined by GC-MS analysis were either below the quantitation limits or ~50% of nominal concentrations (25 or 100 ng/L nominal concentrations, prior to the addition of fish). Of note is that all bioassays and the GC-MS^2 values for 17β-E2 in npH_2O were all similar (19 $E2Eq_{KBluc}$ ng/L, 17 $E2Eq_{YES}$ ng/L, and 18.8 17β-E2 ng/L by GC-MS^2, respectively) indicating the true loss of the chemical is likely.

Results from the bioassay of the EE2 spike sample were all similar (Table 2) with 31, 27 and 34 ng/L (E-Screen, T47D-KBluc assay, and YES, respectively) of the 30 ng/L nominal concentration. These reflect recoveries ranging from 83 to 113% by

Table 4 – Water sample characterization and chemical analysis of estrogens by GC-MS[2].

Bioassay	Relative Estrogen Potency			
	E1	αE2	βE2	E3
T47D-Bluc	1.4	0.007	1	0.23
E-Screen	0.012	0.017	1	0.15
YES	0.410	0.025	1	–

			Concentration ng/L[a]			
Land use	Event type	Water flow (m^3/s)	Estrone (E1)	17α-estradiol (αE2)	17β-estradiol (βE2)	Estriol (E3)
VA cattle grazing 3/12/09	Baseflow	0.007	E 1.4	1.1	–	–
VA cattle grazing 5/11/09	Runoff	0.008	–	–	–	–
WI cattle grazing 8/24/10	Baseflow	0.038	–	–	–	–
WI cattle grazing 6/23/10	Runoff	0.075	0.8	1.0	<0.8	E 0.3
IN cattle grazing 10/1/09	Baseflow	0.0006	1.9	–	–	–
IN cattle grazing 12/9/09	Runoff	0.634	–	–	–	–
IA confined beef 9/29/10	Baseflow	0.153	–	–	–	–
IA confined beef 11/12/10[b]	Runoff	0.120	–	–	–	–
MI dairy 4/2/09	Baseflow	0.056	–	–	–	–
MI dairy 5/14/09	Runoff	0.104	–	–	–	–
WI dairy 5/13/10	Runoff	1.557	3.5	2.1	–	–
WI dairy 9/7/10	Baseflow	0.309	–	–	–	–
NY dairy 11/2/10	Baseflow	0.088	–	–	–	–
NY dairy 11/17/10	Runoff	0.850	8.1	3.8	–	–
IA swine-2 8/24/09	Baseflow	0.009	2.5	–	–	–
IA swine-1 1/23/10	Baseflow	0.028	–	–	–	–
IA swine-2 1/23/10	Baseflow	0.095	E 0.3	–	–	–
IA swine-1 3/15/10[c]	Runoff	1.161	1.5	–	–	–
IA swine-2 3/15/10[d]	Runoff	E 2.549	1.5	–	–	–
IN swine 10/1/09	Baseflow	0.0002	–	–	–	–
IN swine post 12/9/09	Runoff	0.603	–	–	–	–
PA swine 3/4/09[e]	Baseflow	0.006	–	–	–	–
NC swine 3/8/10	Baseflow	0.006	–	–	–	–
NC swine 12/13/10[f]	Baseflow	0.005	–	–	–	–
Swine unplanned sample 2009	Unexpected release of swine manure	nm[g]	298	12.2	E 59.2	E 2.1
MD poultry 3/23/09	Baseflow	0.012	–	–	–	–
MD poultry 5/18/09	Runoff	0.018	1.4	–	–	–
ARK poultry 12/8/10	Baseflow	0.004	–	–	–	–
ARK poultry 3/9/11	Runoff	0.270	–	–	–	–
KY poultry 12/1/10[h]	Runoff	0.0002	1.1	–	–	–
KY poultry 6/20/11	Runoff	0.009	–	–	–	–
OH control 4/02/09	Baseflow	0.004	0.9	–	–	3.2
OH control 5/14/09	Runoff	0.283	–	–	–	–
PA control 3/4/09	Baseflow	0.010	–	–	–	–
PA control 5/14/09	Runoff	0.034	–	–	–	–
IA control 9/29/10	Baseflow	0.017	–	–	–	–
IA control 11/12/10	Runoff	0.016	E 0.5	–	–	–
IN control row crop 11/1/09	Baseflow	0.019	–	–	–	–
IN control row crop 12/9/09	Runoff	0.351	–	–	–	–
			LQ < 0.8	LQ < 0.8	LQ < 0.8	LQ < 2

a Values reported as less than the limit of quantitation (<LQ) designated as dashes; E – estimated values, included in calculations of 17β-E2 chemical equivalence.
b Minimal runoff.
c No sample for E-Screen, snow melt.
d Estimated flow, not amenable for measurement, snow melt.
e No manure applied this year.
f Drought, no runoff, baseflow only.
g Not measured.
h No manure applied this year, soil too wet.

Table 5 – POCIS sample characterization and estrogen analysis by GC-MS[2].

Land use	Estrogen concentration[a] (ng/POCIS per 30 day period)				Deployment			Water flow[b] (m^3/s)
	Estrone (E1)	17α-Estradiol (αE2)	17β-Estradiol (βE2)	Estriol (E3)	Deployed	Retrieved	Days deployed	
VA cattle grazing	10.9	0.8	3.4	–	5/11/2009	6/10/2009	30	0.007–0.008
WI cattle grazing	2.4	1.7	0.3	–	5/25/2010	7/7/2010	42	0.038–0.075
IN cattle grazing	0.4	–	–	–	11/10/2009	12/22/2009	42	0.0006–0.634
IA confined beef[c]	–	–	–	–	10/13/2010	11/17/2010	35	0.153–0.120
MI dairy	1.6	–	–	–	5/14/2009	6/18/2009	35	0.056–0.104
WI dairy	0.6	–	0.2	–	4/14/2010	5/26/2010	42	0.309–1.557
NY dairy	0.9	0.4	–	–	11/2/2010	11/30/2010	28	0.088–0.850
IA swine-1	–	–	–	–	11/19/2009	1/15/2010	88	0.028–1.161
IA swine-2	–	–	–	–	11/19/2009	1/19/2010	92	0.009–2.549[d]
IN swine	0.4	–	–	–	10/1/2009	12/22/2009	42	0.0002–0.60
PA swine[e]	0.2	–	–	–	3/18/2009	5/6/2009	49	0.006
NC swine[f]	–	–	–	–	3/4/2010	4/13/2010	40	0.005–0.006
MD poultry	3.1	0.3	0.8	0.2	5/1/2009	6/2/2009	32	0.012–0.018
ARK poultry	1.6	1.1	–	–	3/3/2011	4/14/2011	28	0.004–0.270
KY poultry[g]	0.5	–	–	–	3/15/2011	5/16/2011	61	0.0002–0.009
IN control	–	–	–	–	11/10/2009	12/22/2009	42	0.019–0.351
OH control	2.3	–	0.2	2.4	5/14/2009	6/18/2009	35	0.004–0.940
PA control	0.2	–	0.1	–	3/23/2009	5/6/2009	44	0.101–0.034
IA control	–	–	–	–	10/13/2010	11/17/2010	35	0.016–0.017
	LQ < 0.2	LQ < 0.2	LQ < 0.2	LQ < 0.5				

a Values reported as les than the limit of quantitation (<LQ) designated as dashes.
b Flow rate at deployment and retrieval.
c Minimal runoff.
d Estimated flow, not amenable for measurement, snow melt.
e No manure applied this year.
f Drought, no runoff, baseflow only.
g No manure applied this year, soil too wet.

bioassay, while chemical analysis for the EE2 spike was 52% of the nominal concentration (15.5 ng/L).

All bioassays had a linear range of response of between 1 and 2 order's of magnitude. The linear range of responses was similar for E-Screen and T47D-KBluc, starting at $\sim 3 \times 10^{-13}$ M 17β-estradiol on plate, but starting ~10 times higher for the YES assay ($\sim 3 \times 10^{-12}$ M on plate). The EC_{50} for the various estrogens and the relative estrogenic potential (REP) in comparison to 17β-E2 are reported in Table 3. It holds true for all three bioassays, that the shape or slope of the curves for each estrogen are not compound specific, but differ only by the concentration at which the biological response is initiated (data not shown).

3.3. *E2Eq of water samples relative to land use*

The concentration of 17β-E2 equivalents from bioassay ($E2Eq_b$) in the 38 water samples ranged from below detection limits to high values that were dependent on the bioassay used, but all were less than 10 ng/L:4.6 [T47D-KBluc, Cattle Dairy NY runoff (RO)]; 2.8 [YES, Swine IA-2 8/09 (BF)]; and 1.4 ng/L [E-Screen, Cattle Grazing VA (BF)] (Fig. 1A–D, Table S2). For biological context, the proposed predicted No Effect Concentration (pNEC, Young et al., 2004) for 17β-E2 in fish is 1 ng/L, with a proposed Lowest Observable Effect Concentration (pLOEC) of 10 ng/L. Caldwell et al. (2012) recently derived a slightly higher pNEC of 2 ng/L for 17β-E2. The original pNEC for 17β-E2 was exceeded both by E-Screen and T47D-KBluc in the VA Cattle Grazing baseflow sample (~1.3 ng/L E2Eq from bioassay ($E2Eq_b$), Fig. 1C). In addition, six of 14 water samples from sites with dairy waste applications or grazing cattle present had estrogenicity from 0.7 to 4.6 ng/L in the T47D-KBluc assay (Table S2). The samples with the greatest $E2Eq_b$ runoff (WI 2.9 and NY 4.6 $E2Eq_{KBluc}$ ng/L) were both collected after field application of dairy manure followed by periods of substantial runoff (flow rates of WI and NY dairy runoff of 1.58 and 0.85 m^3/s, respectively, Table S1). Of note is that these E2Eqs when multiplied by the flow rates for both of the samples, produce similar estimated loads of ~4000 ng/s. Many factors impact runoff concentrations including land management practices, soil type, and slope (Ritter et al., 2002). Soils with silt loam surfaces are dominant in both locations, though the Wisconsin basin has generally shallower soils and steeper slopes (12–20%) with greater potential to generate surface runoff than the NY basin (typically <10% slopes). Obviously direct comparisons are not possible, as concentrations of estrogens in the manures, time and rate of manure applications, and subsequent precipitation and runoff events would have been different at the two sites.

The only other sites with $E2Eq_b$ at or slightly above even the pNEC for 17β-E2, were two poultry sites (MD RO 1.2 on 5/18/09 and KY RO 12/1/10 1.0 $E2Eq_{KBluc}$, Fig. 1D). The flow rates at the time of collection for these samples were ≤ 0.02 m^3/s in both cases. These sites and their condition at the time of sampling differ in several ways. The stream sampled at the KY site had stopped flowing after a prolonged dry period, and rain was required for the December 2010 sampling to take place (e.g. stream to start flowing again). Subsequently, heavy rainfalls

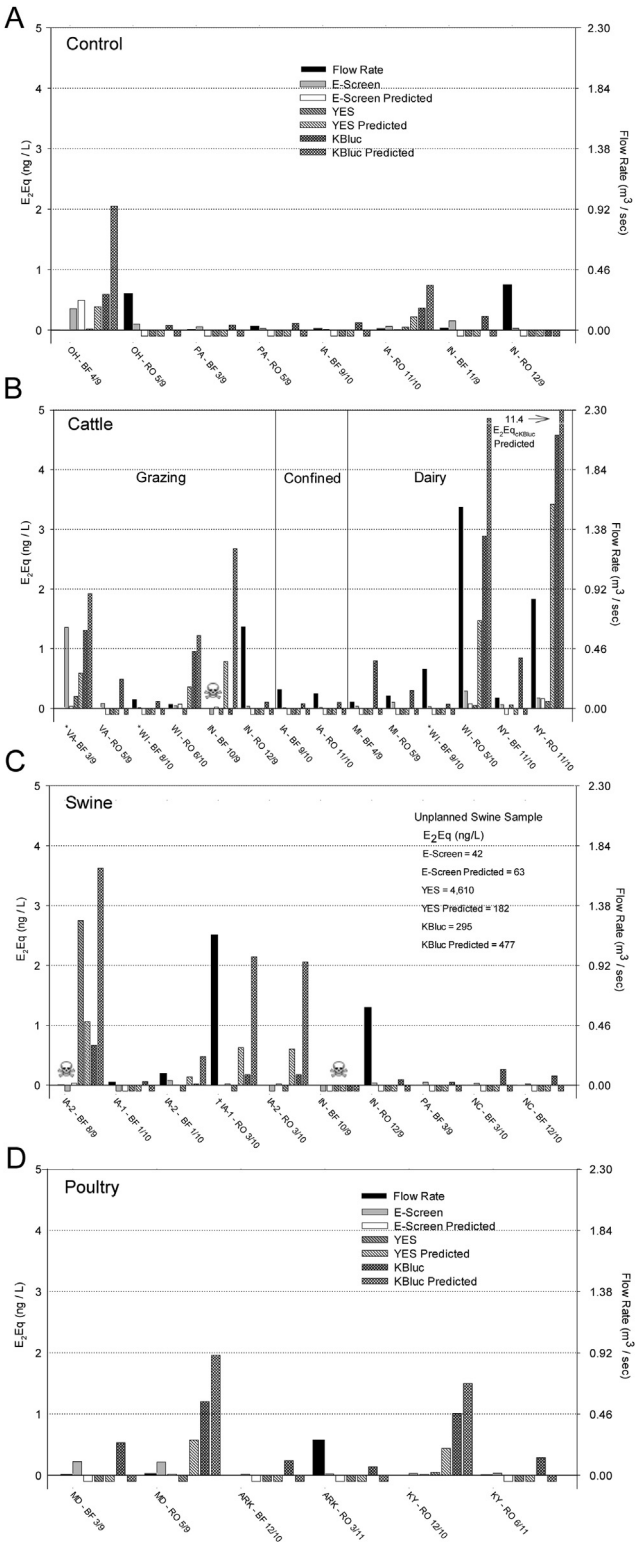

Fig. 1 – Bioassay of discrete stream water samples by land use. Estradiol equivalents (E2Eq) of water sample extracts were determined by E-Screen, YES, or T47D-KBluc, and their respective predicted E2Eq equivalents based on GC-MS^2 analysis × REP's for estrogens. Samples were collected under baseflow (BF) or runoff (RO) conditions. When no activity was detected, values were graphed as negative values. The right Y axis indicates flow rate of the stream at the time of sampling. The proposed predicted No Effect Concentration for 17β-E2 in fish is 1 ng/L and the proposed lowest observable effect concentration is 10 ng/L (Young et al., 2004). Overall coefficients of variations (mean ± SD) were 16% ± 6.9% for E-Screen (n = 5 wells per sample), 12% ± 9.5% for T47D-KBluc (n = 4–8 wells per sample; most were 5 wells in two experiments) for T47D-KBluc, 0.8% ± 0.32% for YES (n = 3 wells per sample). Panel A) Site without livestock operations. B) Sites with cattle operations, note Predicted T47D-KBluc (KBluc) is off graph at 11.4 ng/L (☠) indicates sample toxicity to E-Screen assay cells, here in the baseflow sample from Indiana. C) Sites with swine operations. † IA Swine-1 RO 3/10 denotes no sample for E-Screen. Inset data is from an unplanned sample in response to an unexpected release of swine manure from a storage lagoon. D) Sites with poultry operations. Details of sampled watersheds and manure application/animal access * are provided in Table 1. See Table S2 for numeric values of predicted E2Eqs.

during the spring of 2011 resulted in wet conditions that prevented the application of poultry litter so that the June 2011 water sample was not post-manure application as planned. While the KY site sample collected in June 2011 was during a time of runoff, the flow rate was still very low. In contrast, the MD sample collected during baseflow was pre-application of litter, and the runoff sample was post-litter application. Despite these differences, the estrogenicity of the December 2010 KY runoff sample was nearly identical to the May 2009 MD runoff sample ($E2Eq_{KBluc}$). The source of the estrogenicity in the KY sample is uncertain. The KY basin is approximately twice the size of the MD basin, with an equivalent number of birds reared per year (~480,000). The predominant soil (~60%) upstream at the KY site is Loring silt loam (Oxyaquic Fragiudalf), which has a fragipan horizon that impedes drainage and increases the likelihood that runoff is generated during periods of heavy precipitation. Under extended drought conditions, it might be possible that estrogens present in the previous year's application of litter were still in place, and transported in the December runoff. Other possible explanations for the estrogenicity of the KY water sample include contributions from septic systems (4 were present) or wildlife. If wildlife was the source, the estrogenicity of fresh animal excrement might be expected to have longer residence times in cooler temperatures (KY sample collected in December) than in warmer temperatures (later sample collection was in June). In contrast to the KY basin, the MD basin was very flat and composed of ~40% very poorly drained Corsica mucky loam (Typic Umbraquult) and 40% Hambrook and Woodstown sandy loam soils (Typic and Aquic Hapludults). As a result of a combination of soil types and the level topography, the soil in this area is frequently saturated and drainage ditches are necessary to remove excess water so that the fields can be used for crop production. Post-manure application runoff probably contributed to increased estrogenicity of the water sampled. The increase in the estrogenicity of the MD post-application runoff ($E2Eq_{KBluc}$) versus pre-application sample, however, was not reflected in the $E2Eq_{E\text{-}Screen}$, most likely

because of the lower REP of estrone in the E-Screen compared to the T47D-KBluc assay as detailed previously (Section 3.1).

Of note are the $E2Eq_b$ results on the unplanned water sample collected in response to an unexpected release of swine manure from a storage lagoon (Fig. 1C insert values). The $E2Eq_b$ were extremely variable for this water sample (42, 295, and 4610 ng/L $E2Eq_b$ for E-Screen, T47D-KBluc and YES, respectively), yet both the E-Screen (67%) and T47D-KBluc (62%) assays were in fairly close agreement with their respective predicted E2Eq based on GC-MS^2 data (Table 4). In contrast, the E2Eq from YES were 25 times the predicted E2Eq based on the YES REPs and GC-MS2 data ($E2Eq_{cYES}$).

Water sample results from one control site were unexpected. The Ohio baseflow sample collected on 4/2/09 (Fig. 1A) was found to contain estrone and estriol (Table 4), as well as $E2Eq_b$ by all bioassays. Researchers had made note of the presence of some domestic ducks at this site during sample collection. The potential impact of water fowl provides a frame of reference for potential impact from area wildlife. For contextual comparison, a survey of 11 Minnesota lakes found an average of 0.2 ± 0.16 ng/L of 17β-E2 and 0.5 ± 0.53 ng/L E1, including five lakes with concentrations reported as less than the quantitation limit (<LQ, 0.05 ng/L; Writer et al., 2010). The estrogenic activity found in all samples by E-Screen was confirmed as ER-dependent through co-incubations with the ER receptor agonist ICI 182,780, which prevented cellular proliferation.

The relative sensitivities of the assays (quantitation limits) were evident in the percent of samples that were reported as <LQ. The respective percentages of water samples that were <LQ were 68% by GC-MS^2 and 5%, 14% and 78% for the T47D-KBluc assay, E-Screen, and YES assays, respectively. Although the GC-MS^2 method reported some samples as <LQ, for those samples with ≥0.1 ng/L E2Eq by E-Screen assay the T47D-KBluc assay detected equivalent or higher E2Eqs.

E-Screen failed to detect estrogenic activity in two water samples in which estrogens were detected by the GC-MS^2 analysis. These were IA Swine-2 baseflow 8/09 and IN Cattle baseflow, for which sample toxicity on the E-Screen assay cells was evident (denoted by skull and crossbones on Fig. 1B and C). Estrone concentrations were 2.5 ng/L in IA Swine-2 baseflow 8/09 and 1.9 ng/L for IN cattle baseflow (Table 4). Sample toxicity to assay cells was found in only one other sample: the IN Swine baseflow sample collected on the same date as the IN cattle baseflow, although no estrogens were detected by GC-MS^2 analysis. Of these 3 E-Screen samples with toxic properties (on which no detection of estrogenic activity was possible), estrogenicity was detected in one sample by the other bioassays: the IA Swine1 baseflow sample (2.75 and 0.66 ng/L by the YES and T47D-KBluc assays, respectively), yet the T47D-KBluc value was <20% of predicted by $E2Eq_{KBluc}$ (3.6 ng/L), perhaps a reflection of cellular toxicity.

Because of the differences in relative estrogen potencies of the bioassays, the $E2Eq_b$ for the various assays would not be expected to be in quantitative agreement. Of the 7 samples that were estrogenic in the YES assay, E2Eqs for three were quantitatively similar to E-Screen results (NY Dairy baseflow and runoff, Fig. 1C; and IA control runoff, Fig. 1A, Table S2), while two YES values were about 7 times lower (VA Cattle baseflow, WI Dairy runoff), and one was 18 times lower (Ohio Control baseflow) than the E-Screen.

3.4. *E2Eq of POCIS samples and land use*

Similar trends seen among the bioassays of discrete water sample results were also present for passive water (POCIS) sample results (Fig. 2A–D, Table 5). Of 19 POCIS samples, 32% were <LQs by GC-MS^2, 0% for T47D-KBluc assay, 5% for E-Screen, and 26% for YES assays. Compared to the discrete water samples, detections of the estrogens by GC-MS^2 or estrogenic activity detection by bioassay was dramatically enhanced in POCIS samples, because the POCIS samples represents an integrated sample normalized for a 30 day deployment, which can provide higher resultant extract concentrations. GC-MS^2 analysis of POCIS samples identified estrogens present at five sites where estrogens had not been detected (<LQ) in discrete water samples, with estrone present in all cases, and with 17α-E2 and17β-E2 each also found at one site. These five sites included one control (PA), two swine sites (PA and IN), and one poultry site (AR). There was one exception where the GC-MS^2 of the POCIS sample did not detect the presence of estrogens found in the discrete water sample. This was at the IA Control site, where estrone was detected in the discrete water sample during a runoff event, although at only an estimated 0.5 ng/L.

Comparing bioassay results for discrete water samples to POCIS samples, the relative estrogenicity by site for a particular land use was similar in those cases when POCIS samples were deployed during the period of discrete water sampling. For example, the Ohio site had the highest $E2Eq_b$ of the control sites in both POCIS and discrete water samples, with one exception (a YES discrete water sample that was near the detection limit). In addition to E1 and E3 detected in the discrete water sample from this site (Table 4), 17β-E2 was detected in the POCIS sample (Table 5) (possibly due to the domestic ducks present at this site, though a septic system could not be ruled out as another source of estrogens). For select sites, the discrete samples were taken proximal to the time of the passive sampler deployment, but not within the actual deployment period, making direct comparisons problematic. If a particular unique discharge or rain event occurred during the time of discrete-water sampling, estrogens present in that sample might not be reflected in estrogens present (or not) in the POCIS sampler. In the case of sites with cattle, the $E2Eq_{KBluc}$ of the two WI and NY Dairy runoff discrete water samples (Fig. 1C) were the highest, yet the activity found in the POCIS samples were somewhat similar for all but the VA Beef Grazing sample, which was ≥16 times the other samples (Fig. 2C). This might reflect the cattle's access to the stream above the sampling point, contributing a direct source of estrogens, versus a pulsatile surge in estrogens with runoff events following application periods. Although the POCIS samples chemicals during episodic or pulsatile events, the data are less reflective of maximum concentrations that occur during high flow events, as it represents a time-weighted average concentration.

Reports of higher values of E2Eq in surface waters associated with farming operations from Australia and the United Kingdom (Matthiessen et al., 2006), might reflect differences in

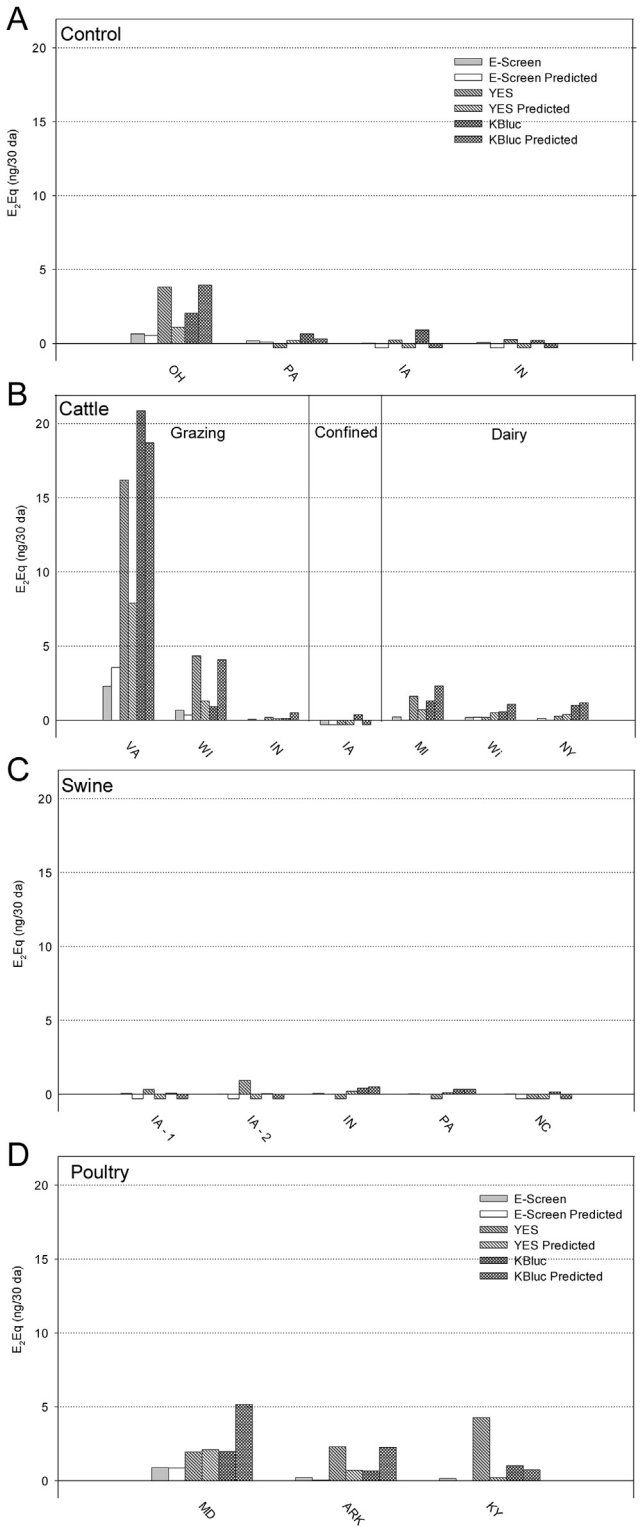

Fig. 2 – Bioassay of extracts from POCIS deployed in streams with various land uses. Estradiol equivalents (E2Eq) of POCIS extracts were determined by E-Screen, YES, or T47D-KBluc assay, and their respective predicted E2Eq based on GC-MS^2 analysis × REP's for estrogens. Values are adjusted for a 30-day deployment period. When no activity was detected, values were graphed as negative values. Coefficients of variations were 12% ± 5.8% for E-Screen (n = 5 wells), 10% ± 9.5% for T47D-KBluc (n = 4 wells per sample in two experiments), and 0.9% ± 0.81% for YES (n = 3 wells). Details of sampled watersheds are provided in Table 1. See Table S3 for numeric values of bioassay and predicted E2Eqs.

livestock production, more similar to the data found at the site with a larger number of grazing cattle (VA). For comparison, analyses of 21-day deployment of POCIS in various Minnesota lakes detected mean concentrations of 0.2 ± 0.18 ng/POCIS for 17β-E2 and 1.0 ± 0.54 ng/POCIS for E1 with two lakes having values <0.2 ng/POCIS of both compounds (Writer et al., 2010). Use of POCIS versus discrete water sampling allowed for higher rates of detection (three more lakes were identified as containing estrogens) in their study, as observed in our study. Of note is that the quantity (ng/POCIS) of estrogens detected in streams across land uses in this study, were typically lower than those reported in the MN lakes, even with longer deployment periods (adjusted to 30 days versus 21 days in the study of lakes).

In the POCIS samples, the $E2Eq_{KBluc}$ were ~5 times the $E2Eq_{E\text{-}Screen}$, again likely a reflection of the greater response to E1 in theT47D-KBluc assay. The estrogenicity values by YES, were greater (~10 times) than those from E-Screen when activity was detected, but were only ~3 times T47D-KBluc values, indicating additional activity in the YES assay, possibly due to chemicals other than steroidal hormones. Of note is the highest $E2Eq_b$ for all the assays were found in samples where GC-MS^2 detected quantifiable amounts of three of the four natural estrogens (E1, 17α-E2,17β-E2, and E3). Estrone and 17β-E2 were present in all of these samples. The bioassays' responsiveness were especially noteworthy when considering the concentrations of these compounds were ≤11 ng/POCIS for E1 and ≤3.4 ng/POCIS for 17β-E2 (per 30 day normalized deployment).

The mirroring of the discrete water and POCIS results indicate that even at low concentrations found in discrete water samples, POCIS will sorb estrogens and reliably indicate sites where estrogens are present at concentrations detectable by GC-MS^2. In POCIS samples, $E2Eq_{KBluc}$ of ~1 ng/POCIS for a 30 day deployment were found at all sites where discrete-water samples were ≥0.6 ng/L $E2Eq_{KBluc}$, with only one exception, the IA Swine-2 site 8/09 where the discrete sample was toxic to E-Screen assay cells. Sites with the lowest POCIS E-Screen results also had the lowest discrete-water $E2Eq_{E\text{-}Screen}$.

3.5. *Predictive $E2Eq_c$ versus measured $E2Eq_b$*

3.5.1. *Practical considerations prior to data assessment*

In an effort to evaluate an assay's ability to accurately predict estrogenicity, results from chemical analyses (like the GC-MS^2 method applied here) are multiplied by the assay's REP for each estrogenic compound and then summed, providing a predicted E2Eq ($E2Eq_c$). The validity of adding the E2Eq for estrogenic compounds is dependent on documentation of an assay's additive versus antagonistic, competitive, or synergistic responses. While several laboratories have compared mixtures of estrogenic compounds to single component

exposures in bioassays to validate their additive nature (e.g., Thorpe et al., 2006), the test concentrations do not always reflect typical environmental concentrations of the compounds. For instance, estrone is typically present in treated animal wastewater at more than 100 times the concentrations of 17β-E2, making detection of 17β-E2 by chemical analysis difficult by comparison (Hutchins et al., 2007).

In contrast, Bermudez et al. (2012) tested the T47D-KBluc assay using defined mixtures of environmental estrogens based on concentrations found in domestic animal and sewage treatment effluents. While the observed and predicted 17β-E2 response values generally fell within the 95% confidence interval, response curves were not superimposable for three of the four mixtures. In part, this might stem from the ratios of the less potent estrogens to E1, the most active compound in this bioassay. The observed E2Eq response was less than predicted when the ratio of E1 to the molar sum of other estrogens was 1:11.4 (this mixture was formulated to provide all estrogens at equipotent concentrations). When the E1 concentration was approximately equivalent to the sum of the molar concentrations of 17α- and β-E2 (dairy mixture, 1:1) the observed response was slightly greater than predicted; and when E1 was in a 4:1 ratio with the molar sum of 17α- and β-E2 and E3 (poultry mixture) the predicted and observed responses were essentially superimposable. The swine mixture presented alterations in both the low and high end of the response curve, with the observed response greater than predicted at low concentrations and vice versa at higher concentrations (ratio of 5:1 E1 to sum of 17β-E2 and E3). In the E-Screen, we have repeatedly observed lower than expected $E2Eq_b$ when 17β-E2 was added to a sample that has high concentrations of estrone (unpublished data). These findings again point to the care with which results should be interpreted and comparisons made.

Because the REPs vary among bioassays, an $E2Eq_c$ should be provided for each bioassay in the laboratory where it is being performed. Furuichi et al. (2006) used this method in evaluating the estrogenicity of swine wastewater along the treatment process. Using the MVLN transactivation assay [MCF-7 breast cancer cells (M) transfected with Xenopus vitellogenin A2 gene (V) controlling the firefly luciferase structural gene (L), transfected clones selected by resistance to neomycin (N) assay], crude extracts and fractionated samples were analyzed by LC-MS^2 for 11 estrogenic compounds in addition to bioassay. The respective REPs (determined in their laboratory) were applied to the chemical data, summed, and compared to the bioassay E2Eq ($E2Eq_b$) value for both the raw extract and the sum of the fractions. The $E2Eq_b$ of the raw extracts reflected only 16–77% of the summed $E2Eq_b$ for the fractions. The lower activity seen in the raw extracts could be due to several factors: fractionation of the samples removed toxic compounds or matrix components that specifically or non-specifically interfered with access to the ER; and/or competition among estrogenic chemicals for the ER was removed by fractionation. For example, competition by 17β-E2 and E3 for active sites would be removed by fractionating the sample, as they are eluted in different fractions than estrone. No mention was made of the assessment of cellular toxicity in their paper. When the $E2Eq_b$ of the sum of the fractions was compared to $E2Eq_c$, only 35–84% of the $E2Eq_b$ were accounted for by the chemical analysis, and the authors concluded that the unknown estrogenic activity was the result of some chemical that had not been identified (in spite of analyzing for an exhaustive list of estrogenic chemicals). If instead the $E2Eq_c$ of the raw extract had been compared to the $E2Eq_b$ raw unfractionated extract, the "unaccounted for" activity would have been greatly reduced.

3.5.2. *Results of bioassays versus predictions based on chemical analysis*

The E-Screen bioassay typically gave greater E2Eqs than the predicted $E2Eq_{cE\text{-}Screen}$. The relative sensitivity of the GC-MS^2 method versus E-Screen would explain most of this, as the LQ for the E-Screen was ~0.03 ng/L E2Eq versus the LQs for the GC-MS^2 of 0.8 ng/L for E1, 17α-E2, and 17β-E2; and 2 ng/L for E3. One might expect that extraction of larger water volumes would overcome detection limits by chemical analysis. However, extracting larger volumes can also increase the concentrations of other components in the matrix that can interfere with compound detection. At concentrations of ≥0.1 ng/L the $E2Eq_{E\text{-}Screen}$ was less than the predicted $E2Eq_{cE\text{-}Screen}$ for only two samples, and they were still ~2/3 of the predicted value (unplanned sample in response to the unexpected release of swine manure from a storage lagoon 67%, Fig. 1B; Ohio Control baseflow, 72%, Fig. 1A). Explanations for the lower $E2Eq_{cE\text{-}Screen}$ could include: the presence of toxic compounds in the samples and/or non-specific matrix interferences that block access to cellular ERs, as well as competition between estrogens as previously discussed (Section 3.5.1). In contrast, most of the E2Eq determined in the T47D-KBluc assay were less than the $E2Eq_{KBluc}$. Of the 13 discrete water samples in which estrogens were detected by GC-MS^2, eight samples averaged 57 ± 16% of predicted $E2Eq_{cKBluc}$. Four others that were all from the same location (IA Swine-1 & IA Swine-2) ranged from 4 to 18% of predicted, (4, 8, 9, and 18% of chemically predicted; Fig. 1C), perhaps reflecting some site-specific sample toxicity to assay cells. Differences in extraction efficiencies could also contribute to differences between bioassay and GC-MS^2 results. Only one sample had no estrogenic activity by T47D-KBluc assay (IN Cattle baseflow – toxic by E-Screen). Similarly, while YES failed to detect estrogenic activity in nine samples that had estrogens by GC-MS^2, observed $E2Eq_{YES}$ were substantially less than $E2Eq_{cYES}$ (2, 5, and 19% of predicted) with one exception. The IA Swine-2 sample from 8/24/09 had $E2Eq_{YES}$ that was 255% of predicted (2.8 versus 1.1 ng/L). In contrast, in the case of the unplanned water sample collected in response to the unexpected release of swine manure from a storage lagoon, where concentrations of estrogens were very high, the YES was more than 24 times the $E2Eq_{cYES}$, indicating a less than satisfactory performance of this assay for use as a sentinel screen.

As a percent of predicted $E2Eq_c$ for POCIS samples, the $E2Eq_{KBlucs}$ were the closest with an average of 78% $E2Eq_{cKBluc}$, while the E2Eq from E-Screen and YES were higher than predicted (actual were 244 and 376% of predicted, respectively). These findings are in contrast to those for YES by Liscio et al. (2009) that found good agreement between YES E2Eq and the $E2E2Eq_{cYES}$, with assay results ranging from 50 to 157% of $E2Eq_{cYES}$ for input and output wastewater samples. The most reasonable explanation for the better agreement observed in

their study, is that higher concentrations of estrogens were found in the effluent downstream from a wastewater treatment plant, compared to those found in our study of surface waters associated with agricultural usage. Their study found 9 of 12 POCIS samples contained ≥20 ng/POCIS of E1 and 6 of 10 samples had ≥10 ng/POCIS of 17β-E2. In our study, only 1 of 19 samples contained >3 ng/POCIS of either E1 or 17β-E2. This again, points to the inability of YES to accurately assess estrogenic activity at lower concentrations. More in line with our findings, were those reported by Matthiessen et al. (2006), comparing POCIS E2Eq to chemically predicted E2Eq on water samples from livestock farms.

4. Conclusions

- Evaluation of U.S. streams at 19 sites for estrogens by chemical analyses (GC-MS^2) and E2Eqs by bioassay analyses found concentrations to be consistently below the United Kingdom proposed Lowest Observable Effect Concentration for 17β-estradiol in fish, (no EE2 present).
- The low detection limits of the E-Screen and T47D-KBluc bioassays provided greater sensitivity than GC-MS^2 and YES.
- The higher relative estrogen potency of estrone in the T47D-KBluc assay makes it the assay of choice to ensure detection of this most prevalent natural estrogen, but might overestimate the overall estrogenic potential for biological effects on fish.
- The E-Screen provided good agreement with the E2Eq$_c$, but all bioassays should be monitored for failure because of cellular toxicity from samples, requiring further evaluation.
- The YES assay provided the least satisfactory assessment of estrogenic activity.
- Bioassay of discrete-water samples or passive water (POCIS) samples can provide for more sensitive detection of estrogens at ~50% of the cost of GC-MS^2 analysis.
- POCIS were an effective tool for the assessment of estrogens at a given site, even allowing the relatively less-sensitive YES bioassay to detect activity.
- Higher estrogenic activity was frequently associated with manure application to fields, but in some instances the increased activity might have been related to the presence of domestic ducks, or delayed estrogen transport by runoff because of dry periods following manure application.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.watres.2013.03.028.

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