SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 34, No. 2, pp. 437–445, 2015

Printed in the USA

Hazard/Risk Assessment

SOURCES OF ENDOCRINE-DISRUPTING COMPOUNDS IN NORTH CAROLINA WATERWAYS: A GEOGRAPHIC INFORMATION SYSTEMS APPROACH

Dana K. Sackett,*† Crystal Lee Pow,‡ Matthew J. Rubino,§ D. Derek Aday,† W. Gregory Cope,† Seth Kullman,‡ James A. Rice,† Thomas J. Kwak,‖ and Mac Law#

†Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina

‡Department of Biological Sciences,, Program in Environmental & Molecular Toxicology, North Carolina State University, Raleigh, North Carolina

§North Carolina Cooperative Fish and Wildlife Research Unit, Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina

‖US Geological Survey, North Carolina Cooperative Fish and Wildlife Research Unit, Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina

#College of Veterinary Medicine, Department of Population Health and Pathobiology, North Carolina State University, Raleigh, North Carolina

(Submitted 9 July 2014; Returned for Revision 17 October 2014; Accepted 31 October 2014)

Abstract: The presence of endocrine-disrupting compounds (EDCs), particularly estrogenic compounds, in the environment has drawn public attention across the globe, yet a clear understanding of the extent and distribution of estrogenic EDCs in surface waters and their relationship to potential sources is lacking. The objective of the present study was to identify and examine the potential input of estrogenic EDC sources in North Carolina water bodies using a geographic information system (GIS) mapping and analysis approach. Existing data from state and federal agencies were used to create point and nonpoint source maps depicting the cumulative contribution of potential sources of estrogenic EDCs to North Carolina surface waters. Water was collected from 33 sites (12 associated with potential point sources, 12 associated with potential nonpoint sources, and 9 reference), to validate the predictive results of the GIS analysis. Estrogenicity (measured as 17β-estradiol equivalence) ranged from 0.06 ng/L to 56.9 ng/L. However, the majority of sites (88%) had water 17β-estradiol concentrations below 1 ng/L. Sites associated with point and nonpoint sources had significantly higher 17β-estradiol levels than reference sites. The results suggested that water 17β-estradiol was reflective of GIS predictions, confirming the relevance of landscape-level influences on water quality and validating the GIS approach to characterize such relationships. *Environ Toxicol Chem* 2015;34:437–445. © 2014 SETAC

Keywords: Ecotoxicology Endocrine disrupting compound Estrogenicity Geographic information system prediction Surface water

INTRODUCTION

The presence of endocrine-disrupting compounds (EDCs) such as estrogens and estrogen mimics in aquatic systems and biota has drawn public concern [1,2]. Evidence linking EDCs with fish reproductive impairment and increased susceptibility to infection indicate that waters impacted by EDCs may lead to fish population declines [3,4]. However, one of the many troubling aspects of EDCs is that we currently lack a clear understanding of the individual- and population-level impacts on the fish themselves or on the wildlife and humans that may consume fish and drink the contaminated water [5]. An important 1st step toward addressing these knowledge gaps and developing mitigation strategies is understanding the extent and distribution of EDCs in surface waters. This information is also critical for identifying potential geographic "hot spots" of health concern that may require remedial action.

Endocrine disruption occurs when an "exogenous chemical interferes with the production, release, transport, binding, action or elimination of natural endogenous hormones" [1]. This can result from a vast number of compounds; however, much of the current aquatic research on EDCs has focused on chemicals that interfere with the estrogen receptor [4,6]. These chemicals can include natural estrogens (e.g., 17β-estradiol), synthetic estrogens (e.g., 17α-ethinylestradiol), and estrogen mimics (e.g., bisphenol A, 4-nonylphenol), all of which have been associated with a high incidence of testicular oocytes (intersex) and the expression of vitellogenin in male fish, a yolk precursor produced in the liver that is stimulated by estrogen (typically seen only in females) [7,8]. The primary sources of natural and synthetic estrogens to the aquatic environment are municipal and agricultural waste flows. Therefore, water bodies downstream of wastewater-treatment facilities and areas with high agricultural runoff have been strongly associated with these estrogenic effects [9,10]. Vajda et al. [10], for example, documented gonadal intersex, altered sex ratios, reduced gonad size, and vitellogenin production in male fish downstream of sewage-treatment facilities. A recent comprehensive study reported widespread incidence of intersex fish in US water bodies [11]. Of particular concern in North Carolina, USA, is that the highest recorded rate (64%–91% of collected fish) of intersex largemouth bass (*Micropterus salmoides*) in that national study occurred in the Yadkin-Pee Dee River, which originates in and flows through the state. Moreover, in many cases fish and other wildlife have been suggested to serve as sentinels for environmental and public health, particularly in relation to EDCs [12,13]. Indeed, EDCs have been putatively linked with the increased prevalence of a number of human diseases and undesirable population trends [5,6,13,14].

Despite the prevalence of estrogenic effects on fish and wildlife, little is known about the extent and distribution of these chemicals in surface waters or how environmental concentrations relate to potential sources of estrogenic EDCs. The objective of the present study was to identify and examine

All Supplemental Data may be found in the online version of this article.

* Address correspondence to danas4@hawaii.edu.

Published online 5 November 2014 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.2797

potential inputs of estrogenic EDC sources in North Carolina water bodies using a geographic information system (ArcGIS 10; ESRI) mapping and analysis approach.

MATERIALS AND METHODS

The location and relative size of estrogenic EDC sources, including wastewater-treatment facilities (both sewage and industrial), confined animal feeding operations (e.g., swine, poultry, cattle), agricultural applications of municipal biosolids, and Superfund sites as putative sources of estrogenic EDC contamination (e.g., polychlorinated biphenyls [PCBs]) [15,16], were incorporated into our GIS analysis to create point and nonpoint source maps depicting the potential inputs of estrogenic EDCs across North Carolina. This approach allowed us to identify water bodies throughout the state that have the potential to be significantly impacted by 1 or more sources of estrogenic EDCs and aided in the identification of relatively unimpacted water bodies, with few or no apparent estrogenic EDC sources of contamination. Our approach assessed numerous potential diffuse sources of estrogenic EDCs at the landscape level. We also sampled and tested the estrogenicity of surface waters throughout North Carolina to determine the validity of our GIS analysis and to examine the extent of estrogenic compounds in surface waters across the state.

Point source map

As part of the National Pollution Discharge Elimination System and North Carolina Department of Environment and Natural Resources (NCDENR) regulations, a permit is required for any wastewater discharge in North Carolina. The 2010 data from these maximum permitted discharges were acquired through the NCDENR (C. McNutt and M. Fleahman, NCDENR, Raleigh, NC, USA, personal communication) and used along with 2011 US Environmental Protection Agency (USEPA) Superfund site data to create a map depicting the potential input of estrogenic EDCs to water bodies, based on the number and relative size of estrogenic EDC sources. The types of wastewater facilities included in the analysis were municipal sewage and industrial discharges. Of sites with industrial discharge, those with $\leq$10% of effluent from human waste treatment were examined further to determine if the facility was likely to discharge any chemicals previously identified as an estrogenic EDC (Supplemental Data, Table S1). If not, then it was censored from our database (e.g., a pickle company with 100% industrial discharge). In addition, data on treatment type at each wastewater facility were unavailable. However, personal communication with personnel at NCDENR (C. McNutt and M. Fleahman, NCDENR, Raleigh, NC, USA, personal communication) suggested that, although there is a range of treatment types across the state, the majority were equipped with tertiary treatment.

To measure the potential impact of these discharges on water bodies in North Carolina, we used the maximum permitted volume of discharge for each facility, measured in thousands of gallons of permitted discharge per day (Figure 1). Actual permitted discharge data were not available. This metric was used as a measure of relative facility size and was summed and $\log_e$-transformed for each 12-digit hydrologic unit code [17] in North Carolina to determine the relative potential impact of the estrogenic EDC sources within small sub-basins (Figure 2). Active Superfund sites (i.e., those not considered "cleaned up" by the USEPA) were also included in the analyses. However, only those Superfund sites that had been contaminated by chemicals putatively linked to endocrine disruption (e.g., DDT, PCBs, mercury [18–20]) were included in our database. In addition, because there was no measure of the relative size of the potential estrogenic EDC input at each Superfund site, we used the average $\log_e$-transformed maximum permitted discharge from the NCDENR database as a measure of the potential estrogenic EDC input. Our original database of estrogenic EDC point sources included 1289 records; following adjustment (only including records putatively linked to sources of estrogenic endocrine disruption), the database included 1121 records (Figure 1). Sources summed by each 12-digit hydrologic unit code were classified into color-coded groups ranging from low to high using natural breaks in the data in GIS (Figure 2). Further, summing the relative estrogenic EDC input by each 12-digit hydrologic unit code provided inherent standardization because a 12-digit hydrologic unit code is the 6th and smallest level of watershed subdivision by the US Geological Survey, representing similarly sized subwatersheds.

Nonpoint source map

The NCDENR 2010 permit data for animal operations and the agricultural application of municipal biosolids from wastewater-treatment facilities were used to create a nonpoint source map depicting water bodies with the potential input from estrogenic EDCs based on the number and relative size of estrogenic EDC sources. Relative facility size was measured using the permitted number of animals allowed at a particular location. Although coarse, this measure estimated the relative estrogenic EDC impact of each facility on surface waters. This measure was $\log_e$-transformed and used to characterize the potential input of the facilities within each 12-digit hydrologic unit code. In addition, because there was no measure of the relative size of potential estrogenic EDC input at each site where municipal (human) waste was applied to the land and only relative measures for agricultural sites were available, the average $\log_e$-transformed permitted number of animals was used as a measure of potential input for those locations (Figure 2). As with our point source map, the summed nonpoint source impact for each 12-digit hydrologic unit code was also classified into color-coded groups ranging from low to high using natural breaks in the data in GIS (Figure 2).

Water sampling and analyses

Following development of both point and nonpoint source maps, we identified 33 sites across North Carolina to sample water for assessment and validation; 12 were associated with potential point sources, 12 were associated with potential nonpoint sources, and 9 were reference sites that were not associated with any known or suspected contaminant source (Figure 3). These sites were chosen to span from low to high in potential estrogenic EDC inputs based on our mapped and color-coded designations (Figure 2), to span various ecoregions, and by accessibility to sampling sites. In addition, although we attempted to make sample locations in hydrologic unit codes where only point or nonpoint sources were present and succeeded in most cases (63% of sample sites were taken in a hydrologic unit code with only point or nonpoint sources present), a few did have both sources present. In those rare cases, we only chose sample locations where the predicted impact for the hydrologic unit code in the opposing map (e.g., nonpoint source designation for a point source sample) was relatively low ($<$23 $\log_e$[permitted discharge] and $<$ 23 $\log_e$[permitted animals]; see Figure 2) and chose a sample site that was as far upstream of the undesired source as access would allow but also downstream of at least 1 of the target

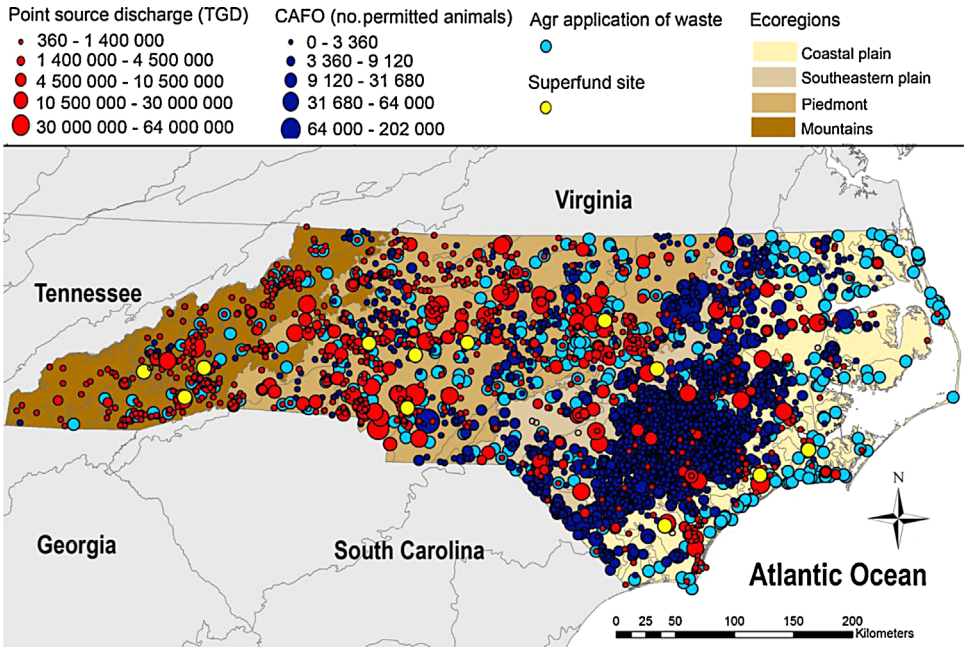

Figure 1. Data, compiled from the National Pollutant Discharge Elimination System, North Carolina Department of Environmental and Natural Resources, and US Environmental Protection Agency, used to identify locations and relative size of potential estrogenic endocrine-disrupting compound sources. Ecoregions are indicted on the map by background shading [25]. TGD = thousands of gallons per day; CAFO = concentrated animal feeding operation; Agr = agriculture.

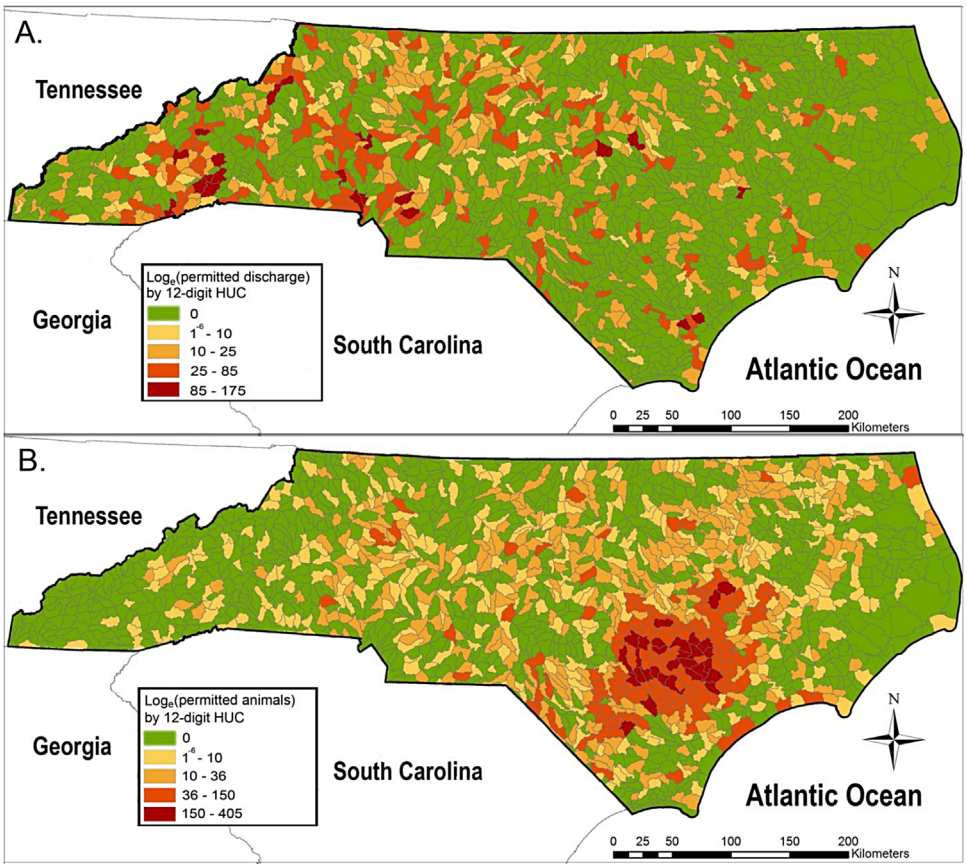

Figure 2. Potential inputs of sources of estrogenic endocrine-disrupting compounds across North Carolina from (**A**) point (i.e., wastewater-treatment facilities and Superfund sites) and (**B**) nonpoint (i.e., concentrated animal feeding operations, agricultural application of municipal waste; Figure 1) sources. Inputs were measured by permitted discharge in thousands of gallons per day for point sources and by permitted number of animals for nonpoint sources. These inputs were summed by 12-digit hydrologic unit codes. Dark red and orange colors indicate higher relative inputs. HUC = hydrologic unit code.

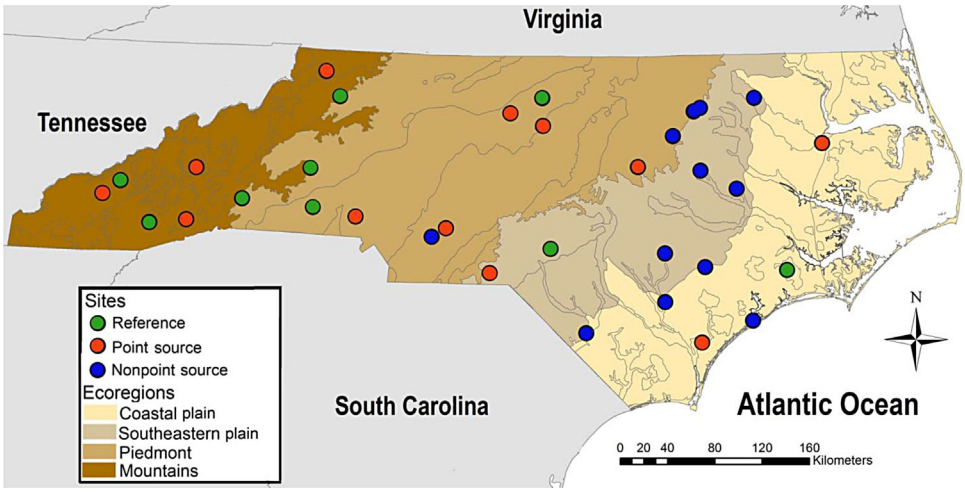

Figure 3. Water sampling sites where estrogenic activity was measured. Twelve of these sites were associated with point sources of estrogenic endocrine-disruption compounds (EDCs), 12 were associated with nonpoint sources of estrogenic EDCs, and 9 were not associated with any sources based on the present data (reference; Figure 1). Ecoregions are indicted on the map by background shading [25].

source types. At each site, grab water samples (2 L) were collected by hand from surface waters in precleaned 2-L amber bottles during fall and winter (November 2011–February 2012) and extracted using Oasis HLB (Waters) solid-phase extraction cartridges. Compounds of interest were eluted using consecutive elutions of dichloromethane, methanol, and acetone. Mean recovery of solid-phase extraction from laboratory deionized water spiked with 17β-estradiol was 112.3% (standard error = 13.5%). Sample extracts were concentrated up to approximately 2000 times in ethanol and stored at –20°C until analysis with the yeast estrogen screen bioassay. The yeast estrogen screen bioassay was performed according to the method of Routledge and Sumpter [21] and modified by Chen et al. [22]. Briefly, 200 μL of sample extract were added to a 96-well plate and serially diluted 1:2 in a solution of 90% water and 10% ethanol (12 dilutions total). Each sample was run in duplicate on the same plate. The estrogen 17β-estradiol served as a dose–response standard and was added to a separate row on the same plate from a standard solution prepared in 10% ethanol. The serially diluted samples and EEQ standard were allowed to incubate with yeast solution (yeast nitrogen base, peptone, and sucrose) for 3 d. On day 3, an assay buffer containing ortho-nitrophenyl-β-galactoside (Calbiochem) was added, producing a colorimetric response at 405 nm. The reaction was stopped with 200 μL of a 1-M sodium carbonate solution, and the plates were centrifuged at 875 g for 10 min. A portion (100 μL) of the resulting supernatant was taken from each well and transferred to a new 96-well microtiter plate to determine the optical density (OD) at 405 nm and 620 nm.

Data analysis for yeast estrogen screen assays was conducted as follows: for each dilution point, OD 620 was subtracted from OD 405 to account for colorimetric interference from yeast cells. This value was then normalized to the negative control. The sigmoid concentration–response curve of the 17β-estradiol standard was fitted to a symmetric logistic function using GraphPad Prism software (GraphPad). The responses of the standard and the sample were expressed as a percentage of the maximum response evoked by 17β-estradiol, and the concentration of 17β-estradiol that induced a half-maximal response (i.e., 50% effective concentration [EC50]) was then fitted using the software. For each sample, the concentration factor of sample extract that induced a half-maximal response was also fitted using the software (50% concentration factor [CF50i). The estrogenic activity of each sample, expressed as 17β-estradiol equivalents (EEQ), was then calculated as $EEQ = EC50/CF50_i$, where EC50 is the observed half-maximal estrogenic activity of the 17β-estradiol standard and $CF50_i$ is the observed half-maximal estrogenic activity of sample *i*. Using these EEQs, an equivalent concentration for each sample was calculated as Equivalent 17β-estradiol concentration = EEQ × 272.83, where 272.83 is the molecular weight of 17β-estradiol. The 17β-estradiol response was consistent across all assay plates, producing a mean EC50 of 98.2 ng/L with a standard error of 3.9 ng/L.

Statistical analyses

Water samples taken within 12-digit hydrologic unit codes were hypothesized to be associated with potential point sources, nonpoint sources, and no sources (reference) of estrogenic EDCs. Previous research suggested that many estrogenic EDCs degrade relatively quickly in water and concentrations would be expected to decline as distance from a source increased [23,24]. However, because the objective of the present study was to discern the landscape-scale influx of estrogenic EDCs into surface waters and because accessibility to sample sites in each hydrologic unit code were not an equal distance from the closest source, we did not control for the distance each water sample was taken from the closest estrogenic EDC source (e.g., wastewater-treatment plant) in each hydrologic unit code. It was then necessary to standardize EEQ data by the distance each sample was taken from the closest source. To standardize these data, we measured the shortest linear distance from the source to the sample site along the waterway 5 times in ArcGIS. The EEQ data were then divided by the mean of these 5 measurements and multiplied by the mean of all distances measured. This standardization ensured that differences in EEQ values were not driven by distance from the closest source within the hydrologic unit code (e.g., the sample taken closest to an estrogenic EDC source had the highest EEQ concentration) in the analyses and, thus, were more representative of the average concentration in the hydrologic unit code and not a single source. The mean and standard deviation for distances from sample sites to the closest point and nonpoint sources were 2.47 ± 2.39 km, excluding reference sites. With reference sites these values were much higher, as would be expected (7.44 ± 9.40 km). All data were $\log_e$-transformed to meet

assumptions of normality and equality of variance for parametric tests. Comparisons of water estrogenicity data among source type (nonpoint, point, and reference) were conducted using one-way analysis of variance. To determine whether our mapping approach was predictive of estrogen activity in water, we compared EEQ values to relative estrogenic EDC inputs predicted by our GIS analysis using 3 candidate standard least squares linear regression models. These models included the predicted input from our GIS map, the ecoregion where the sample was taken [25], and both of these parameters together. We included ecoregion (coastal plain, southeastern plain, piedmont, and mountain) in our models because of its common use among agency biologists and because these specified regions can stratify the environment by its probable response to disturbance [26]. These models were tested for each map (point and nonpoint source). Model significance was determined with analysis of variance ($p < 0.05$), and model fit was compared using the adjusted regression coefficient (adjusted R^2). Although map predictions were categorical, we ran models treating these data as ordinal and then again as continuous to ensure the relation with predicted input was not an artifact of data treatment. All statistical analyses were conducted using JMP software (Ver 8.0; SAS Institute).

In addition, EEQ values were compared with the levels deemed "safe" (<1 ng/L) and "high risk" (>10 ng/L) by the Environment Agency of the United Kingdom [27,28]. We used these criteria because the USEPA does not currently have criteria by which to compare estrogenicity from mixtures of estrogenic EDCs in water to adverse affects on aquatic life. The USEPA has a criterion for a single type of estrogenic EDC (ethinyl estradiol [29]). However, there are numerous other estrogenic compounds in water bodies that may contribute to estrogenic effects observed in aquatic life [30].

RESULTS

Our GIS analysis suggested that point and nonpoint source contributions of estrogenic EDCs were distributed somewhat regionally across the state, with nonpoint source inputs concentrated in southeastern North Carolina and point source contributions concentrated in central and western North Carolina (Figure 2A and B). Concentrations of estrogen activity in water sampled from across the state, measured as EEQ, ranged from 0.06 ng/L to 56.9 ng/L. The concentrations at a majority of the sites (29 of 33, 88%) were below 1 ng/L (range, 0.06 ng/L–0.9 ng/L). Of the remaining sites, 9% (3 of 33) were between 1 ng/L and 10 ng/L (range, 1.1 ng/L–4.3 ng/L, 2 of which were from point source sites and 1 from a reference site) and only 1 site (3%) was above 10 ng/L (56.9 ng/L, a point source site), measures deemed intermediate and high risk by the Environment Agency of the United Kingdom [27,28]. Further, all of the nonpoint sources had EEQ concentrations <1ng/L. However, water samples associated with point and nonpoint sources had significantly ($p < 0.01$) higher concentrations of estrogenic activity compared with reference sites (Figure 4).

Model analyses showed that our point and nonpoint source map predictions successfully explained variation in estrogenic activity of surface water at the landscape scale ($p = 0.01$; Table 1). Relative predicted input from our point source map explained 38% of the variation in EEQ data and showed an increase in estrogenic activity with increased relative input predicted by our GIS analysis (Figure 5A). Although the point source model including predicted input and ecoregion was

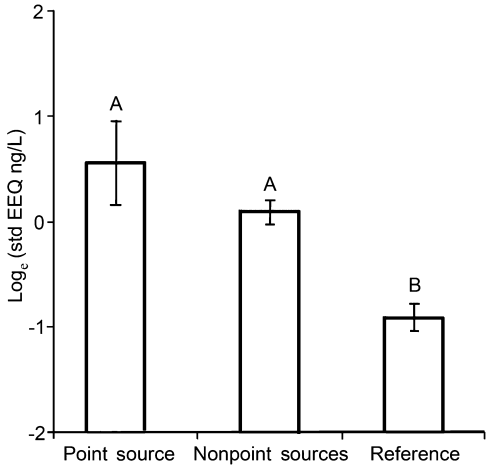

Figure 4. Mean and standard error (error bars) of water estrogenic activity (EEQ) from sites associated with point sources of estrogenic endocrine-disrupting compounds (EDCs), sites associated with nonpoint sources of estrogenic EDCs, and reference sites associated with no sources of estrogenic EDCs based on the present data (Figure 2). Different capital letters signify a statistically significant difference in means ($p < 0.05$). Std = standardized.

significant ($p = 0.01$, adjusted $R^2 = 0.54$) and accounted for additional variation in the data, ecoregion alone was only marginally significant in this model ($p = 0.07$), with the southeastern portion of the state having the highest predicted estrogenic EDC concentrations. Relative predicted input from our nonpoint source map explained 66% of the variation in the data and showed an increase in estrogenic activity with increased relative input (Table 1 and Figure 5B). Similar to the results for the point source map, the nonpoint source model including both ecoregion and predicted input was significant and explained more variation in the data ($p < 0.01$, adjusted $R^2 = 0.72$); however, again ecoregion in this model was not significant ($p = 0.15$). We also identified an outlier in our database, a sample taken at a reference site that had much higher estrogenic activity than expected (EEQ = 1.12 ng/L, mean of other reference sites = 0.29 ng/L). After further on-the-ground investigation as well as evaluating the analytical run for this sample, we found no known source that could explain the higher concentration at this site. Removal of this outlier from the database did not drastically change the fit of either point ($p = 0.01$, adjusted $R^2 = 0.40$) or nonpoint ($p < 0.01$, adjusted $R^2 = 0.74$) source models with predicted input alone. Ecoregion was, however, elevated to significance ($p = 0.02$) in the point source model including both predicted input and ecoregion when the outlier was excluded ($p < 0.01$, adjusted $R^2 = 0.63$). Further, treating predicted input data as a continuous variable did not change the overall results; though it did slightly reduce regression coefficients (Table 1). Flow varied widely among sample sites (the range in the 2-yr recurrent peak flow was 2–1206 m^3/s [31]). However, flow was not significantly different among point, nonpoint, and reference sites ($p = 0.14$), did not relate to water estrogenicity ($p = 0.99$), and despite the wide range, a significant relationship between water estrogenicity and predicted input was still evident.

DISCUSSION

Our GIS analysis applied readily available permit data to identify water bodies impacted and unimpacted by estrogenic EDCs throughout North Carolina, informed water sampling

Table 1. Model parameters and regression statistics for 3 standard least squares regression models developed to explain water estrogenic activity at sites associated with point and nonpoint sources

	Ordinal[b]			Continuous[b]	
	n	*p*	R^{2c}	*p*	R^{2c}
Point source models					
Ecoregion, predicted input[a]	21	0.01	0.54	<0.01	0.52
Predicted input[a]	21	0.01	0.38	0.01	0.30
Ecoregion	21	0.20	0.10	0.20	0.10
Nonpoint source models					
Ecoregion, predicted input[a]	21	<0.01	0.72	<0.01	0.69
Predicted input[a]	21	<0.01	0.66	<0.01	0.66
Ecoregion	21	0.06	0.23	0.06	0.23

[a]Inputs are predicted by the number and relative size of potential sources of estrogenic endocrine-disrupting compounds to the sampled water body and are color-coded in Figure 2.
[b]Models were run with the factor "predicted input" treated as ordinal and continuous.
[c]Values represent the adjusted R^2.

locations, and provided the foundation for future investigations of estrogenic EDC effects on resident aquatic biota. Despite the relatively course metrics and conservative approach used in the present study, our point and nonpoint source maps were predictive of relative estrogenic activity in surface waters at a landscape scale. Although there are numerous other demographic factors that can impact the level of estrogenic EDCs in surface waters, particularly on a scale smaller than that of the present study (e.g., number of human females in the local population, particularly those using oral contraceptives like ethinyl estradiol, local population size, sewage treatment type, the operational age and condition of a treatment plant, pesticide use, and water chemistry [32,33]), many of these are difficult to measure and data are not readily available. In addition, although compromised septic systems are a potential source of estrogenic EDCs, these data were unavailable for the present study as well as for others [34], signifying that better records of the location and condition of septic systems should be made available. Some other models have used more detailed information to predict the inputs of estrogenic EDCs on water bodies [35], however, because those models incorporated factors more difficult to compile, their utility and reproducibility for state and federal resource management agencies and other decision makers are somewhat limited. Here, we used only data that were available from state and federally mandated permits to create maps and then validated that they are predictive of water estrogenicity. Further, while hydrological data have also been used in the past, our approach of using the EEQ concentrations (ng/L) from the receiving waterbody and not the effluent, takes into account discharge and to some degree the degradation of estrogenic EDCs with distance from the source. We suggest that this type of predictive mapping is a necessary step for studies on estrogenic EDCs to better understand the potential inputs and sources of estrogenic EDCs across large geographic areas.

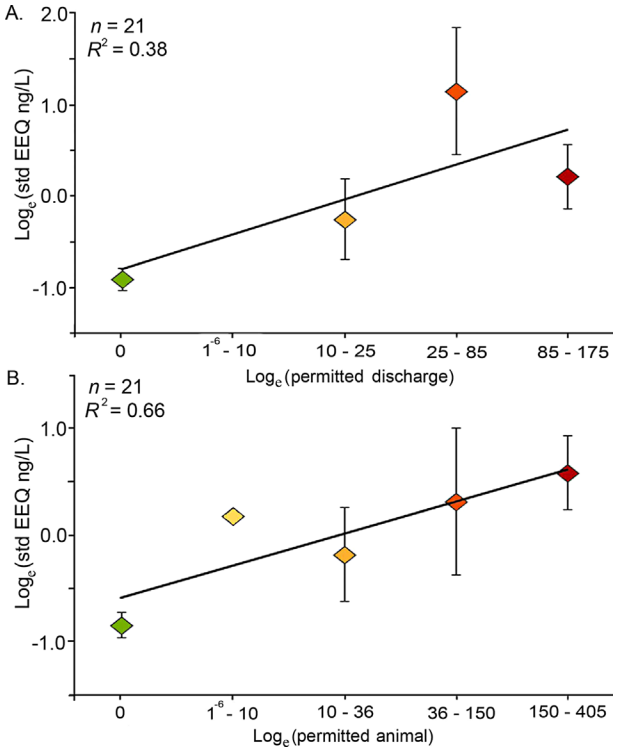

Figure 5. Mean and standard error (error bars) of estrogenic activity (EEQ) by predicted inputs (based on the permitted discharge and permitted number of animals) from (**A**) point and (**B**) nonpoint source maps (Figure 3). Ordinal and continuous models were significant ($p < 0.01$) in both cases (Table 1). Ordinal model results are depicted. The sample size for each model was 21. Equations for regression lines in these figures are (**A**) $y = 0.38x - 1.18$ and (B) $y = 0.30x - 0.88$. The R^2 values were adjusted R^2 values. Colors represent predicted input levels in Figure 2. Std = standardized.

Estrogenic EDCs in water

The majority (88%) of EEQ concentrations measured across the state were below 1 ng/L, a level considered to hold no risk for aquatic species by the Environment Agency of the United Kingdom [27,28]. Of the remaining sites, 9% were considered of intermediate risk and only 1 site (3%) was considered high risk. All but 1 of these higher-risk sites were associated with point sources. Indeed, the site with the highest concentration was the same site in Hinck et al. [11] from the Yadkin-Pee Dee River in North Carolina, which was associated with some of the highest percentages of intersex largemouth bass sampled across the nation. However, recent research suggests that low doses of EDCs found in the environment (below the lowest observable adverse effect level) may indeed cause negative effects on fish and wildlife [20]. These negative consequences may be a function of nonmonotonic dose–response curves, where the lowest and highest concentrations of EDCs have the most adverse affects [20,36]. As such, additional work is needed to better understand low-dose effects of EDCs on the endocrine and immune systems of aquatic biota, wildlife, and humans.

The higher concentration of estrogenic activity from samples taken in 12-digit hydrologic unit codes with point and nonpoint estrogenic EDC sources suggests that both types of sources affected water concentrations of estrogenic EDCs during fall and winter (when our samples were collected). Although concentrations of EDCs in point source effluent can fluctuate temporally, wastewater-treatment facilities operate continuously, likely releasing estrogenic EDCs on a regular basis [2]. Conversely, nonpoint sources, such as confined animal feeding operations, generally apply animal waste to agricultural land on a seasonal basis as an integral part of the waste-management plan [37]. Because discrete (grab) water samples only represent conditions at the time of sampling and can miss episodic events such as surface runoff from agricultural fields after waste has been applied, we suggest that our samples may not be representative of the year-round concentrations occurring in waters surrounding nonpoint sources. Other studies have reported seasonal changes in EDCs in surface runoff associated with agricultural applications, with the highest levels usually occurring in spring after crop planting [38,39]. Of particular concern are the relatively high detectable levels of estrogenic EDCs in surface waters found during our fall sampling, when surface runoff would likely be less than during other times of the year (e.g., after spring crop planting and fertilization and during summer low flows). Further, point sources are also expected to have slightly higher EDC concentrations in spring than fall, when our samples were taken, because microbes that degrade waste are less efficient in cool spring temperatures [40].

Predicted input

Point and nonpoint source contributions of estrogenic EDCs were regional in occurrence. The majority of nonpoint source inputs occurred in southeastern North Carolina, where much of the row crop and animal agriculture is located, whereas the higher potential inputs from point source contributions were concentrated in central and western North Carolina, home to larger urban and suburban land uses. Model analysis showed that our point and nonpoint source maps were predictive of the relative levels of estrogenic EDCs in surface waters and could be used to identify areas likely to be impacted by these compounds. Although numerous point sources such as wastewater-treatment plants have been associated with estrogenic EDCs in surface waters and effects on fish and wildlife (i.e., vitellogenin production and intersex), these effects are often only associated with 1 or a few point sources [7–10]. The present study examined the cumulative relative impact of estrogenic EDC sources at a much larger scale. Previous studies have also linked elevated levels of estrogens in surface runoff, receiving waters, and groundwater with agricultural applications of organic waste, particularly in relation to confined animal feeding operations [38,41]. For example, Finlay-Moore et al. [42] reported surface runoff EEQ from broiler litter from 50 ng/kg to 2300 ng/kg dry weight waste, whereas adjacent groundwater estrogen ranged from 6 ng/kg to 66 ng/kg dry weight. In other studies, runoff from sites with land application of manure and poultry litter resulted in elevated levels of estrogen in runoff and adjacent streams [43,44]. Despite the fact that these compounds degrade relatively quickly in surface waters (hours to months [23,24]) and our samples were taken a few months following peak seasonal applications of waste [38,39], our map predictions were still reflective of the level of estrogenic compounds in the water. Environmental exposure of low estrogenic EDC concentrations to fish likely occur intermittently over a generation as a result of seasonal applications of waste to agricultural fields, changes in precipitation that can dilute or concentrate estrogenic EDCs, fish movement in and out of areas with estrogenic EDC contamination, or other temporal variation in source influxes. Panter et al. [45] demonstrated that intermittent exposures of estrogens caused a greater response (increased vitellogenin concentration) in male fish than continuous exposure. They also suggested that estrogenic effects would be sustained for long periods after the transient exposure. These results imply that a metric such as fish intersex, which incorporates the long-term and intermittent low-dose exposures that occur in aquatic systems, may be even more reflective of our map predictions than a static water sample taken at a single point in time.

Although ecoregion was only marginally significant in our point source model with both factors (predicted input and ecoregion), the inclusion of this factor did improve the fit of the model. In addition, because most ecoregions for the point source and nonpoint source maps separately had sample sizes less than 5, the lack of significance for ecoregion may have been an artifact of the relatively low sample size across each ecoregion. Regardless, this lack of significance was unexpected because the ecoregions sampled in the present study are classified as vastly different environments that likely respond differently to disturbance and pollutants [26]. In addition, others have demonstrated that differences in water retention and chemistry, similar to those among the ecoregions that we sampled, can impact the concentration of estrogenic EDCs and other aquatic contaminants [46,47]. For instance, the southeastern plain consists of low-elevation, flat plains that are poorly drained, which would be expected to slow the removal of estrogenic EDCs from surface waters in this ecoregion [25]. Also, Liu and Liu [46] demonstrated that photodegradation of estrogen decreased with pH, suggesting lower rates of estrogen removal from surface waters at lower pH values. Water bodies in the southeastern and coastal plain ecoregions are generally more acidic (lower pH) than the mountain and piedmont ecoregions of North Carolina [25,47]. Despite these differences, the present results suggest that ecoregion characteristics did not strongly influence the concentrations of estrogenic EDCs measured in surface waters.

One of our reference sites, located within a 12-digit hydrologic unit code predicted to have little to no estrogenic EDC input, had a relatively high EEQ concentration and no known source that could explain the contamination. We suggest that unrecorded historical contamination (i.e., legacy effects [48]) or a chemical or compound not yet identified as an estrogenic EDC resulted in the higher level of estrogenic activity measured at this site. The removal of this outlier did not change our overall results, suggesting that while our model is robust and relevant for North Carolina and the technique of creating a predictive map is beneficial, sites considered reference (relatively uncontaminated) should be treated with caution and sampled to ensure low estrogenic EDC contamination. Current research is identifying new chemicals and compounds that interact with the estrogen receptor, block the androgen receptor, or alter steroid synthesis [2,49]. For example, a recent study suggested that cyanobacteria in small impoundments may have estrogenic effects on fish [49]. Although our sites were all lotic, in contrary to those lentic systems studied by Kellock et al. [49], their findings suggest that causal relationships among compound or condition and estrogenic effects are complex and that caution should be exercised when identifying reference or relatively uncontaminated sites.

Adverse health effects have demonstrated the need to better manage exposure to estrogenic EDCs for fish and wildlife that directly rely on suitable environmental conditions for reproduction and survival, as well as for implications to the human population [3–6,12–14]. We provide an important 1st step in developing management strategies by reporting the extent and distribution of estrogenic EDCs in surface waters in relation to multiple sources and by demonstrating a landscape-level mapping approach to identify areas with potential relative inputs from estrogenic compounds. The next steps should be to gain a better understanding of the temporal changes of EEQ concentrations across this geographic range in relation to potential sources, particularly with spring crop planting, and to determine how a biotic metric such as fish intersex, which incorporates long-term and intermittent low-dose exposures, relates to spatial predictions based on potential inputs.

SUPPLEMENTAL DATA

Table S1 (14 KB XLS).

Acknowledgment—The present project was funded by the North Carolina Wildlife Resources Commission (NCWRC) through Sport Fish Restoration Funds. We thank the US Environmental Protection Agency, the National Pollutant Discharge Elimination System, and the North Carolina Department of Environmental and Natural Resources (NCDENR) for access to and assistance with data compilation. Special thanks go to C. McNutt and M. Fleahman of NCDENR. Additional thanks go to B. Tracy and C. Cranford of the North Carolina Division of Water Quality; C. Bryan of the Endangered Species Branch at Fort Bragg, NC; and P. Super of the Great Smokey Mountains National Park for assisting with site access and sampling. We also thank E. Yost for help with field sampling and laboratory analysis. The North Carolina Cooperative Fish and Wildlife Research Unit is jointly supported by North Carolina State University, NCWRC, US Geological Survey, US Fish and Wildlife Service, and Wildlife Management Institute. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US government.

REFERENCES

1. US Environmental Protection Agency 1997. Special report on environmental endocrine disruption: An effects assessment and analysis. EPA/630/R-96/012 Washington, DC.
2. Sumpter JP. 2005. Endocrine disrupters in the aquatic environment: An overview. *Acta Hydrochim Hydrobiol* 33:9–16.
3. Nash JP, Kime DE, Van der Ven LTM, Wester PW, Brion F, Maack G, Stahlschmidt-Allner P, Tyler CR. 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ Health Perspect* 112:1725–1733.
4. Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW. 2007. Collapse of a fish population after exposure to a synthetic estrogen. *Proc Natl Acad Sci USA* 104:8897–8901.
5. Edwards TM, Moore BC, Guillette LJ, Jr. 2006. Reproductive dysgenesis in wildlife: A comparative view. *Int J Androl* 29:109–121.
6. Witorsch RJ. 2002. Endocrine disruptors: Can biological effects and environmental risks be predicted. *Regul Toxicol Pharmacol* 36:118–130.
7. Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. 1998. Widespread sexual disruption in wild fish. *Environ Sci Technol* 32:2498–2506.
8. Pait AS, Nelson JO. Endocrine disruption in fish: An assessment of recent research and results. *NOAA Technical Memo NOS NCCOS CCMA 149*. Silver Spring, MD, USA.
9. Blazer VS, Iwanowicz LR, Henderson H, Mazik PM, Jenkins JA, Alvarez DA, Young JA. 2007. Reproductive endocrine disruption in smallmouth bass (*Micropterus dolomieu*) in the Potomac River basin: Spatial and temporal comparisons of biological effects. *Environ Monit Assess* 184:4309–4334.
10. Vajda AM, Barber LB, Gray JL, Lopez EM, Woodling JD, Norris DO. 2008. Reproductive disruption in fish downstream from an estrogenic wastewater effluent. *Environ Sci Technol* 42:3407–3414.
11. Hinck JE, Blazer VS, Schmitt CJ, Papoulias DM, Tillitt DE. 2009. Widespread occurrence of intersex in black basses (*Micropterus* spp.) from U.S. rivers, 1995-2004. *Aquat Toxicol* 95:60–70.
12. Colborn T. 1995. Environmental estrogens: Health implications for humans and wildlife. *Environ Health Perspect* 103:135–136.
13. Harrison PTC, Holmes P, Humfrey CDN. 1997. Reproductive health in humans and wildlife: Are adverse trends associated with environmental chemical exposure. *Sci Total Environ* 205:97–106.
14. Crain DA, Janssen SJ, Edwards TM, Heindel J, Ho S, Hunt P, Iguchi T, Juul A, McLachlan JA, Schwartz J, Skakkebaek N, Soto AM, Swan S, Walker C, Woodruff TK, Woodruff TJ, Giudice LC, Guillette LJ, Jr. 2008. Female reproductive disorders: The roles of endocrine-disrupting compounds and developmental timing. *Fertil Steril* 90:991–940.
15. Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change. *Environ Health Perspect* 107:907–938.
16. Barnes KK, Kolpin DW, Furlong ET, Zaugg SD, Meyer MT, Barber LBA. 2008. A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States—I) Groundwater. *Sci Total Environ* 402:192–200.
17. Seaber PR, Kapinos FP, Knapp GL. 1987. Hydrologic unit maps. US Geological Survey Water-Supply Paper 2294:63.
18. Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R, Sheehan DM. 2000. The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. *Toxicol Sci* 54:138–153.
19. Zhu X, Kusaka Y, Sato K, Zhang Q. 2000. The endocrine disruptive effects of mercury. *Environ Health Prev Med* 4:174–183.
20. Vandenberg LN, Colborn T, Hayes TB, Heindel JJ, Jacobs DR, Jr, Lee D, Shioda T, Soto AM, vom Saal FS, Welshons WV, Zoeller RT, Myers JP. 2012. Hormones and endocrine-disrupting chemicals: Low dose effects and nonmonotonic dose responses. *Endocr Rev* 33:378–455.
21. Routledge EJ, Sumpter JP. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ Toxicol Chem* 15:241–248.
22. Chen P, Linden KG, Hinton DE, Kashiwada S, Rosenfeldt EJ, Kullman SW. 2006. Biological assessment of bisphenol: A degradation in water following direct photolysis and UV advanced oxidation. *Chemosphere* 65:1094–1102.
23. Ying G, Kookana RS. 2003. Degradation of five selected endocrine-disrupting chemicals in seawater and marine sediment. *Environ Sci Technol* 37:1256–1260.
24. Chowdhury RR, Charpentier PA, Ray MB. 2011. Photodegradation of 17β-estradiol in aquatic solution under solar irradiation: Kinetics and influencing water parameters. *J Photochem Photobiol A Chem* 219:67–75.
25. Griffith GE, Omernik JM, Comstock JA, Schafale MP, McNab WH, Lenat DR, MacPherson TF, Glover JB, Shelburne VB. 2002. Ecoregions of North Carolina and South Carolina (color poster with map, descriptive text, summary tables, and photographs). US Geological Survey. Reston, VA.
26. Bryce SA, Omernik JM, Larsen DP. 1999. Ecoregions—A geographic framework to guide risk characterization and ecosystem management. *Environmental Practice* 1:141–155.
27. 2004. Proposed predicted-no-effect-concentrations (PNECs) for natural and synthetic steroid oestrogens in surface waters. Technical Report P2-T04/1. Bristol, UK.
28. Environment Agency. 2008. Catchment risk assessment of steroid oestrogens from sewage treatment works. SC030275/SR3 Bristol, UK.
29. US Environmental Protection Agency. 2008. Aquatic life criteria for contaminants of emerging concern. White Paper, OW/ORD Emerging Contaminants Workgroup. Washington, DC.
30. Gadd JB, Tremblay LA, Northcott GL. 2010. Steroid estrogens, conjugated estrogens and estrogenic activity in farm dairy shed effluents. *Environ Pollut* 158:730–736.
31. US Geological Survey 2014. StreamStats. [cited 2014 October 2]. Available from http://water.usgs.gov/osw/streamstats/north_carolina.html.
32. Ullman JL. 2006. The chemical behavior of estrone and 17β-estradiol in the environment. PhD thesis. Texas A and M University, College Station, TX, USA.
33. Johnson AC, Williams RJ, Simpson P, Kanda R. 2007. What difference might sewage treatment performance make to endocrine disruption in rivers. *Environ Pollut* 147:194–202.
34. Young J, Iwanowicz L, Sperry A, Blazer V. 2014. A landscape-based reconnaissance survey of estrogenic activity in streams of the upper Potomac, upper James, and Shenandoah Rivers, USA. *Environ Monit Assess* 186:5531–5545.

35. Williams RJ, Keller VDJ, Johnson AC, Young AR, Holmes MGR, Wells C, Gross-Sorokin M, Benstead R. 2009. A national risk assessment for intersex in fish arising from steroid estrogens. *Environ Toxicol Chem* 28:220–230.
36. Welshons WV, Thayer KA, Judy BM, Taylor JA, Curran EM, vom Saal FS. 2003. Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environ Health Perspect* 111:994–1006.
37. Centner TJ, Feitshans TA. 2006. Regulating manure application discharges from concentrated animal feeding operations in the United States. *Environ Pollut* 141:571–573.
38. Khanal SK, Xie B, Thompson ML, Sung S, Ong S, Van Leeuwen J. 2006. Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. *Environ Sci Technol* 40:6537–6546.
39. Alvarez DA, Cranor WL, Perkins SD, Schroeder VL, Iwanowicz LR, Clark RC, Guy CP, Pinkney AE, Blazer VS, Mullican JE. 2009. Reproductive health of bass in the Potomac, USA, drainage: Part 2. Seasonal occurrence of persistent and emerging organic contaminants. *Environ Toxicol Chem* 28:1084–1095.
40. Bringolf RB, Summerfelt RC. 2003. Reduction of estrogenic activity of municipal wastewater by aerated lagoon treatment facilities. *Environ Toxicol Chem* 22:77–83.
41. Soto AM, Calabro JM, Prechtl NV, Yau AY, Orlando EF, Daxenberger A, Kolok AS, Guillette LJ, le Bizec, Lange B, Sonnenschein IG. 2004. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska, USA. *Environ Health Perspect* 112:346–352.
42. Finlay-Moore O, Hartel PG, Cabrera ML. 2000. 17β-Estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. *J Environ Qual* 29:1604–1611.
43. Dyer AR, Raman DR, Mullen MD, Burns RT, Moody LB, Layton AC, Sayler GS. 2001. Determination of 17β-estradiol concentrations in runoff from plots receiving dairy manure. ASAE Meeting paper, No. 01-2107, ASAE, St. Joseph p01–2107, MI USA.
44. Shore LS, Correll DL, Chakraborty PK, Steele K. 1995. Relationship of fertilization with chicken manure and concentrations of estrogens in small streams. In Steele K, ed, *Animal Waste and the Land–Water Interface*. Lewis, Boca Raton, FL, USA.
45. Panter GH, Thompson RS, Sumpter JP. 2000. Intermittent exposure of fish to estradiol. *Environ Sci Technol* 34:2756–2760.
46. Liu B, Liu XL. 2004. Direct photolysis of estrogens in aqueous solutions. *Sci Total Environ* 320:269–274.
47. Sackett DK, Aday DD, Rice JA, Cope WG. 2009. A statewide assessment of mercury dynamics in North Carolina water bodies and fish. *Trans Am Fish Soc* 139:1328–1341.
48. Harding JS, Benfield EF, Bolstad PV, Helfman GS, Jones EBD, III. 1998. Stream biodiversity: The ghost of land use past. *Proc Natl Acad Sci USA* 95:14843–14847.
49. Kellock KA, Trushel BE, Ely PC, Jennings CA, Bringolf RB. 2014. Survey of intersex largemouth bass from impoundments in Georgia USA. *Trans Am Fish Soc* 143:565–572.