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Effects of watershed densities of animal feeding operations on nutrient concentrations and estrogenic activity in agricultural streams

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ABSTRACT

Application of manures from animal feeding operations (AFOs) as fertilizer on agricultural land can introduce nutrients and hormones (e.g. estrogens) to streams. A landscape-scale study was conducted in the Shenandoah River watershed (Virginia, USA) in order to assess the relationship between densities of AFOs in watersheds of agricultural streams and in-stream nutrient concentrations and estrogenic activity. The effect of wastewater treatment plants (WWTPs) on nutrients and estrogenic activity was also evaluated. During periods of high and low flow, dissolved inorganic nitrogen (DIN) and orthophosphate (PO_4-P) concentrations were analyzed and estrogens/estrogenic compounds were extracted and quantified as17β-estradiol equivalents (E2Eq) using a bioluminescent yeast estrogen screen. Estrogenic activity was measurable in the majority of collected samples, and 20% had E2Eq concentrations > 1 ng/L. Relatively high concentrations of DIN (> 1000 μg/L) were also frequently detected. During all sampling periods, there were strong relationships between watershed densities of AFOs and in-stream concentrations of DIN ($R^2 = 0.56–0.81$) and E2Eq ($R^2 = 0.39–0.75$). Relationships between watershed densities of AFOs and PO_4-P were weaker, but were also significant ($R^2 = 0.27–0.57$). When combined with the effect of watershed AFO density, streams receiving WWTP effluent had higher concentrations of PO_4-P than streams without WWTP discharges, and PO_4-P was the only analyte with a consistent relationship to WWTPs. The results of this study suggest that as the watershed density of AFOs increases, there is a proportional increase in the potential for nonpoint source pollution of agricultural streams and their receiving waters by nutrients, particularly DIN, and compounds that can cause endocrine disruption in aquatic organisms.

1. Introduction

Livestock wastes contain high concentrations of nutrients and steroidal estrogens (Hanselman et al., 2003; Johnson et al., 2006; Mallin and Cahoon, 2003; USDA, 1992). These compounds may enter surface waters through runoff or leachate from agricultural land that has received applications of manure from animal feeding operations (AFOs) as fertilizer (Finlay-Moore et al., 2000; Kjaer et al., 2007; Matthiessen et al., 2006; Shore et al., 1995). Surface water contamination by nutrients and hormones in animal waste can also occur when grazing animals deposit waste into or directly adjacent to bodies of water (Kolodziej and Sedlak, 2007). The negative effects of excess nutrients in surface waters are well documented (e.g. Boesch et al., 2001). Several studies have shown that estrogens can disrupt endocrine system function of aquatic organisms at concentrations less than 10 ng/L (Young et al., 2004). In regions with high densities of AFOs, there is increasing concern that high rates of manure application on local agricultural land will lead to eutrophication of surface waters and potential endocrine-related effects in aquatic biota (Kellog et al., 2000; Mallin and Cahoon, 2003; Yonkos et al., 2010).

Relationships between AFOs, nutrients, estrogens, and streams require further assessment in order to manage livestock waste effectively and protect the health of surface waters in agricultural landscapes. A recent study of Iowa rivers in watersheds receiving wastes from AFOs showed a strong relationship between nitrate concentrations and watershed densities of animal units within the AFOs (Weldon and Hornbuckle, 2006), but hormone concentrations were not evaluated. Most studies of estrogens or estrogenic activity in livestock wastes have focused on the potential for aquatic contamination, through measurement of concentrations in storage facilities (Hutchins et al., 2007; Raman et al., 2004) or concentrations in runoff and leachate from manure-treated fields (Finlay-Moore et al., 2000; Kjaer et al., 2007; Nichols et al., 1997). In-stream assessments have generally been conducted in headwater streams adjacent to individual feedlots or farms receiving waste applications (Matthiessen et al., 2006; Shore et al., 1995). These studies isolated the effects of animal wastes on concentrations of estrogens in streams, but did not assess the cumulative downstream effects of animal wastes that may occur in streams with larger drainage areas.

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The relationship between livestock production and water quality is of particular interest in the Shenandoah River watershed (Virginia, USA). The 7600 km^2 watershed is 39% agricultural land, which receives manure from approximately 1200 AFOs and 300 farms that maintain grazing beef cattle (VADCR, 2010; VADEQ, 2006). Seasonal fish kills have occurred in the Shenandoah River since 2004, and resident smallmouth bass have impaired immune function (Ripley et al., 2008) and a high proportion of males with intersex compared to other basins (Blazer et al., 2007). Because estrogenic compounds can induce intersex (Hahlbeck et al., 2004; Lange et al., 2009) and affect the immune function of fishes (Iwanowicz and Ottinger, 2009; Robertson et al., 2009), assessment of their presence in the watershed and relationship to land use is warranted. Nutrient concentrations are of concern due to risk of local eutrophication and potential effects on the health of aquatic organisms (Camargo et al., 2005; Guillette and Edwards, 2005; Johnson et al., 2010). The relationship between nutrient concentrations and land use has management implications outside of the Shenandoah River watershed, as the Shenandoah discharges into the Potomac River, a major tributary of Chesapeake Bay. In order to adequately protect and restore the health of Chesapeake Bay, estimated required reductions in nitrogen and phosphorous loadings for the Shenandoah and Potomac Rivers are 44% and 29%, respectively (Commonwealth of Virginia, 2005).

Comprehensive assessment of the potential effect of AFOs on concentrations of estrogens and nutrients in streams within agricultural landscapes requires consideration of additional sources of these compounds. The Shenandoah River watershed has 81 municipal wastewater treatment plants (WWTPs); the majority (71) are minor facilities discharging less than 1 million gal of effluent per day. Effluent from WWTPs can be a significant source of natural and synthetic estrogens from human excretion, and synthetic xenoestrogens from household and industrial use, to surface waters (Lagana et al., 2004; Muller et al., 2008; Petrovic et al., 2002; Ying et al., 2008). Depending on the level of treatment, WWTPs can also contribute significant nutrient loads to receiving waters, and reducing these loads is a significant component of the strategy to protect and restore the health of Chesapeake Bay (Commonwealth of Virginia, 2005).

The main objective of this study was to evaluate relationships between watershed densities of AFOs and concentrations of nutrients and estrogenic activity in streams within the larger Shenandoah River watershed. Increasing in-stream concentrations of nutrients and estrogenic activity were expected with increasing watershed densities of AFOs due to the potential for increased manure application in the local area. A secondary objective was to examine the effect of WWTPs in combination with AFOs on nutrients and estrogenic activity. The presence of WWTPs was expected to further increase concentrations of nutrients and estrogenic activity in streams. Estrogenic activity was selected over measurement of individual compounds in order to assess the overall potential for biological activity of estrogens and estrogenic compounds in stream water, and to evaluate the utility of screening techniques in large-scale water quality monitoring programs.

2. Methods

2.1. Selection and characterization of study sites

Land use in the Shenandoah River watershed was characterized using a geographic information system (ArcGis 9.3, ESRI, Redlands, CA). Delineated 12-digit, 6th level Hydrologic Unit Code (HUC 6) subwatersheds, 40–160 km^2 in size, were used to quantify all land uses within drainage areas of Shenandoah River tributaries. Locations and numbers of animal units for AFOs (poultry, dairy, and beef), farms maintaining grazing beef cattle, and locations and permit information for WWTPs were obtained from Virginia state agencies. In Virginia, AFOs are defined as facilities that confine animals for at least 45 days and preclude the growth of vegetation, while concentrated AFOs maintain >300 animals (cattle or swine). In the Shenandoah River watershed, the 680 poultry AFOs maintain 10,000–200,000 birds and all hold Virginia Pollution Abatement (VPA) permits. Only 21 of 430 dairy AFOs and three of 110 beef AFOs are concentrated operations and require VPA permits. For this study, the 278 farms maintaining grazing beef cattle were included in beef AFO calculations, because of a similar risk of contamination of surface water from manure of grazing animals (Kolodziej and Sedlak, 2007; Soupir et al., 2006). Many pastures in the area have noticeably high concentrations of feces, particularly during winter/early spring, and cattle are allowed access to streams for water during the summer in many areas (personal observation).

Eighteen sampling sites were selected to represent a gradient of influence from AFOs combined with presence/absence of WWTP discharges (Fig. 1). Sampling sites were located in 14 Shenandoah River tributaries; four tributaries drained multiple subwatersheds and an upstream sampling site was located in the primary subwatershed as well as a downstream site draining multiple subwatersheds. Eight sampling sites could not be located near the outlet of the delineated HUC 6 subwatershed due to limited tributary access, and the watershed area was recalculated using U.S. Geological Survey Digital Elevation Model (DEM) data (30 m resolution). The new delineation was used to quantify upstream land use variables. The number of each type of AFO and each type of animal in each watershed was converted to a density (number/1000 acres) and the total watershed density of AFOs (all types) was calculated for each sampling site. For poultry, dairy, and beef AFOs, the watershed density of animals was strongly correlated with the watershed density of each type of operation ($r = 0.96$, 0.99, and 0.88, respectively), so only AFO density was used in data analysis. For each watershed with a WWTP discharge, the WWTP permit information was used to calculate the total permitted effluent discharge (in millions of gallons per day; MGD). Pasture/hay and cropland are primary application sites for manure from AFOs. Therefore, land cover data for the Shenandoah River watershed (30 m resolution) were obtained from the 2001 National Land Cover Database (Homer et al., 2007). Reclassification and areal tabulation were used to quantify the percentage of forest, developed land, pasture/hay, and cultivated crops upstream of each sampling site. Percentages of all other land use types (i.e. open water, barren land, etc.) were negligible (<1%) in each watershed of interest. For each study site, a total of nine land use variables

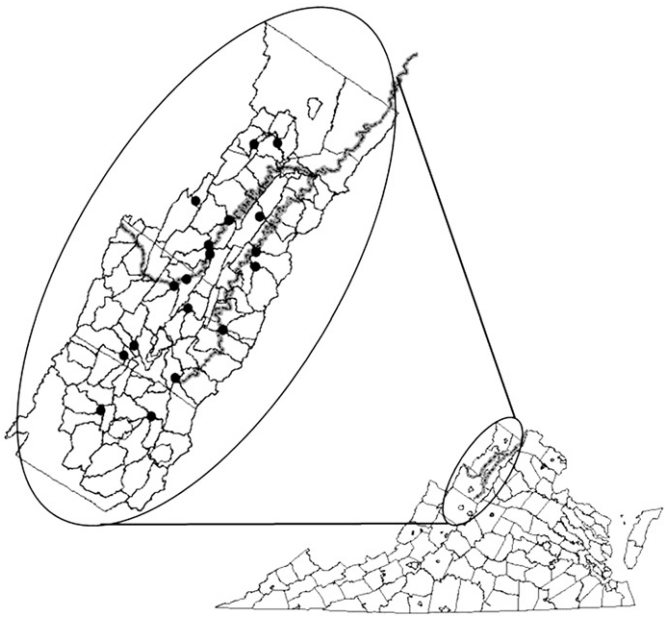

Fig. 1. Locations of the 18 study sites within the Shenandoah River watershed. The enlargement shows the 78 12-digit hydrologic unit code (HUC) subwatersheds of the Shenandoah River (indicated in grey) and the counties included in the entire watershed, relative to the state of Virginia.

were quantified (details provided in Supplementary data). In addition, the presence of any upstream industrial facilities holding a Virginia pollutant discharge elimination system (VPDES) permit was documented for each sampling site (see Supplementary data).

2.2. Field sampling and water quality analysis

Field sampling was conducted in late spring (14–24 May 2008, 21–30 May 2009), mid-summer (8–14 August 2008, 10–11 August 2009), and late winter (10–15 March 2009 only). Late spring represented a period of relatively high flow after the majority of spring manure applications had occurred and mid-summer represented near-baseflow conditions. Late winter was selected to represent a time period prior to spring manure applications, but due to low precipitation, this period also represented relatively low-flow conditions in 2009. Discharge data from the North Fork Shenandoah River (Mount Jackson, VA) illustrate these trends (http://nwis.waterdata.usgs.gov/va/nwis/dv?cb_00060=on&format=gif_default&begin_date=2008-03-01&end_date=2009-10-01&site_no=01633000&referred_module=sw). Sampling was not conducted immediately after rain events. If a rain event occurred during a sampling period, sampling was discontinued until flow at local gauging stations returned halfway back to previous baseflow. Discharge was calculated at each site for all 2009 sampling events using the midsection method (Gore, 2006).

Water samples collected for nutrient analysis were field-filtered using an acid-washed syringe filtration apparatus containing a pre-ashed GF/F filter (0.7 μm). Filtrate was collected in sterile 50 mL polypropylene tubes, preserved with 0.01% v/v chloroform, held on ice, and stored at 4 °C. Within two weeks of collection, dissolved nutrients (NO_3-N, NH_4-N, and PO_4-P) were analyzed using flow injection analysis (Lachat QuickChem 8500, Hach Co., Loveland, CO) following standard methods (APHA, 2005a, 2005b, 2005c). The mean relative percent differences (RPDs) for field duplicates (8 pairs) were 1.3%, 14%, and 8.0% for NO_3-N, NH_4-N, and PO_4-P, respectively. Concentrations of NO_3-N and NH_4-N were summed to total dissolved inorganic nitrogen (DIN) for data analysis. In the majority of samples, NH_4-N concentrations were <5% of total DIN.

Water samples were collected for analysis of estrogenicity using 900 mL pre-cleaned amber glass bottles (I-Chem, Rockwood, TN), acidified to pH 3 with 12.1 N HCl (Havens et al., 2010; Muller et al., 2008; Salste et al., 2007), held on ice, and stored at 4 °C. Within 1 week of collection, the preserved water samples were filtered through a pre-weighed pre-ashed GF/F filter (0.7 μm) using a solvent rinsed all-glass apparatus. Methanol (1–1.5 mL) was used to wash any estrogenic compounds off the retained suspended solids. One blank (HPLC water at pH 3) was filtered with every batch of 10 samples (two per sampling period). Filtered samples and blanks were subjected to solid phase extraction (SPE) using OASIS® HLB (200 mg) glass cartridges (Waters Corporation, Milford, MA), following a modified version of a method published by Lagana et al. (2004). All solvents were HPLC grade. Cartridges were sequentially pre-conditioned with 5 mL each of: ethyl acetate, 50:50 methanol:dichloromethane (DCM), methanol, and HPLC-grade water. Each sample (400 mL) was loaded onto the cartridge at a flow rate of 5–6 mL/min (continuous vacuum). Elution solvents consisted of 6 mL methanol and 6 mL of 50:50 methanol:DCM, which were combined into fraction one. Ethyl acetate (6 mL) was the final elution solvent (fraction two). Eluates were reduced to dryness under ultra high purity N_2 and re-dissolved in 1 mL of methanol. Preliminary analyses of spiked samples indicated no estrogenic activity in fraction two. Therefore, only fraction one was analyzed.

The total concentration of estrogenic compounds in the water samples relative to 17β-estradiol (E2) was estimated using a bioluminescent yeast estrogen screen (BLYES; Sanseverino et al., 2005). The BLYES assay was performed at the U.S. Geological Survey (USGS) National Fish Health Laboratory (Leetown, WV) with strain BLYES provided by Dr. John Sanseverino (University of Tennessee) and maintained in a dormant stage at 4 °C in modified Yeast Minimal Media (YMM leu-, ura-; Routledge and Sumpter, 1996). For the assay, strain BLYES was brought to early stationary phase in YMM at 30 °C on a rotary shaker to an approximate OD_{600} of 0.750. The BLYES assay was performed in sterile, clear-bottom, black polystyrene 96-well assay plates (Costar, Corning Incorporated, Corning, NY). Samples were diluted to 10% in YMM, and 100 μl of the diluted sample was added to each well in duplicate. All assay plates included a 12-point standard curve consisting of E2 ranging from 2.3×10^{-11}–5.0×10^{-8} M and sample blanks containing YMM only. Strain BLYES was added to all preloaded wells at a volume of 100 μl, resulting in a final sample dilution of 5%. Plates (covered) were incubated in the dark at 30 °C for 6 h on an orbital shaker. Luminescence was quantified using a SpectraFluor Plus plate reader (Tecan Group Ltd, Durham, NC), in luminescence mode (1 s integration time/well, gain 180). Luminescence was read over two kinetic cycles separated by 159 s and orbital shaking (5 s). Data from the second kinetic cycle were analyzed.

The BLYES responds to E2 as a standard non-linear dose–response curve (Sanseverino et al., 2005), and produced maximum luminescence from 5.6×10^{-9}–5.0×10^{-8} M E2 after 6 h of incubation. In order to avoid potential errors in non-linear curve fitting at the lower end of the standard curve (where most sample concentrations were located), a linear calibration curve was created using $\log_{10}$ transformations of the five lowest standards (2.3×10^{-11}–2.1×10^{-10} M E2) and their associated mean luminescence. Concentrations in samples with luminescence above this range were quantified using four points from the linear portion of the dose–response curve ($\log_{10}$[E2] vs. mean luminescence; 1.2×10^{-10}–1.9×10^{-9} M E2). Mean luminescence values of zero wells (YMM only) were subtracted from luminescence values of all E2 standards prior to calculation of standard curves. The quantitation limit (QL) of E2 was 2.3×10^{-11} M (0.31 ng/L in samples), luminescence for this standard was always >600 units above that of YMM-only wells. Luminescence of blanks was never above that of the lowest standard, the difference in mean luminescence between blanks and YMM ranged from 82 to 282 units. Mean luminescence of the blanks was subtracted from the raw luminescence for all samples collected on that date (same plate) prior to calculation of sample concentrations. Concentrations were calculated from luminescence in each duplicate well, and the final reported concentration is the mean value. All calculated sample concentrations are reported as ng/L E2 equivalents (E2Eq). For E2Eq concentrations below the QL (<33% of samples during any sampling period), 0.156 ng/L (1/2 QL) was substituted for statistical analyses and load calculations.

Samples of HPLC water and site water (from a site with E2Eq<QL during the first sampling period) were spiked with E2 (100–295 ng) to assess method recoveries. Using the BLYES for quantification, the mean percent recovery of E2 was 93% in HPLC water and 91% in site water. The extracts were also analyzed for E2 at the Virginia Institute of Marine Science (Gloucester Point, VA) using the UPLC/MS^2 method developed by Rice and Hale (2009). The mean percent recovery of E2 was 91% in HPLC water and 90% in site water. Using BLYES, the mean RPD for field duplicates (6 pairs) was 13%. Variability of BLYES assay results was assessed by analyzing 10 samples on two different dates, and the mean RPD was 12% (coefficient of variation was 8%).

2.3. Data analysis

Different types of AFOs co-occurred within the studied watersheds (correlation coefficients for poultry and beef AFOs, dairy and beef AFOs, and dairy and poultry AFOs, were r = 0.87, 0.79, and 0.58, respectively). Therefore, the total watershed density of AFOs was examined as potentially predictive of concentrations and loadings of E2Eq, DIN, and PO_4-P. Relationships between AFO density and analytes were assessed using regression analysis. The interquartile range*1.5 added to, or subtracted from, upper and lower quartiles (25th and

Table 1
Results of linear regressions between watershed densities of AFOs and analyte concentrations and loadings, including proportions of variance explained (R^2; top) and slopes ± standard error (bottom).

	Concentrations			Loadings[a]		
	E2Eq	DIN	PO_4-P	E2Eq	DIN	PO_4-P
May 2008	0.75†† (0.55 ± 0.08)	0.81†† (0.97 ± 0.11)	0.27* (0.43 ± 0.17)			
Aug 2008	0.39** (0.33 ± 0.10)	0.60†† (0.88 ± 0.18)	0.39** (0.68 ± 0.23)			
Mar 2009	0.45** (0.54 ± 0.15)	0.59† (1.1 ± 0.24)	NS	0.23* (0.42 ± 0.19)	0.42** (1.0 ± 0.28)	NS
May 2009	0.63†† (0.55 ± 0.11)	0.78†† (0.95 ± 0.13)	0.57† (0.60 ± 0.13)	0.51† (0.50 ± 0.12)	0.66†† (0.90 ± 0.16)	0.31* (0.47 ± 0.18)
Aug 2009	0.58† (0.57 ± 0.12)	0.56† (0.94 ± 0.21)	0.44** (0.53 ± 0.16)	0.70†† (0.67 ± 0.11)	0.50† (1.05 ± 0.26)	0.44** (0.59 ± 0.17)

NS: regression was not statistically significant ($p \geq 0.05$)
Loadings were not calculated in 2008 because discharge was not measured.
* $p < 0.05$.
** $p \leq 0.01$.
† $p \leq 0.001$.
†† $p \leq 0.0001$: p-values of regression models.

75th percentiles) was used to identify outliers in the dataset and their effect on statistical analyses was tested, as described below. Variables were tested for normality using the Shapiro–Wilk W test. Non-normal data were transformed as follows: $\log_{10}$ for concentration/loading data and square root for density and proportion data. The influence of outliers on regression models was assessed through calculation of Cook's D (Montgomery et al., 2006). Outliers with Cook's D > 0.25 were removed from the model and resulting changes in R^2 and slope were assessed. Outliers were only excluded from final models if their inclusion altered R^2 (change > 10%) and slope (change > 20%) values. This process resulted in exclusion of only one analyte from one site (PO_4-P at Muddy Creek; see Results section).

In order to assess the relative effect of watershed density of AFOs on analyte concentrations and loadings, the R^2 of the final models were compared to R^2 values for regressions of watershed percentages of land cover variables. Colinearity between watershed density of AFOs and watershed percentages of pasture/hay and cropland ($r \geq 0.81$ for all combinations) prohibited the use of multiple regression to assess their potential combined effects. Therefore, an agricultural index (AI) was calculated for each site, where AI = (proportion of pasture/hay + proportion of cropland) * density of AFOs. Relationships between the AI and analyte concentrations/loadings were also assessed using regression analysis and R^2 values were compared with other models.

The potential effects of WWTPs on analyte concentrations/loadings were assessed categorically; the presence or absence of WWTPs in each watershed was utilized as a treatment variable in an analysis of covariance (ANCOVA), as described below. The proportion of stream flow as WWTP effluent was also estimated using discharge data from each sampling site and the total permitted flow of WWTP effluent in the watershed. The relationship between estimated proportions of WWTP effluent and analyte concentrations/loadings was assessed using univariate regression analysis and stepwise multiple regression in combination with total AFO density. However, results did not differ from those of the categorical analysis and are not presented here.

A two-way ANCOVA was used to simultaneously test the effects of WWTP presence and sampling period on analyte concentrations/loadings (y), while taking into account significant relationships with the watershed density of AFOs (covariate, x). Initially, all variables and possible interactions were included in the models. Non-significant interaction terms were sequentially removed until final models with only significant variables were produced (Milliken and Johnson, 1984). If there were no significant covariate–treatment interactions (equal slopes), pairwise comparisons (t-test) of least-squares adjusted means ($X = \bar{x}$) were used to quantify significant treatment effects. If significant covariate–treatment interactions were present (unequal slopes), regression models and least-squares adjusted means were compared at three different points: $\bar{x}$, mean of three lowest x values, and mean of three highest x values (Milliken and Johnson, 1984).

All correlations within independent and dependent variables were evaluated using Pearson product moment correlation analysis. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC), with a significance level of $\alpha = 0.05$.

3. Results

Within the 90 water samples collected during this study, concentrations of DIN ranged from 5.0 to 7588 μg/L. Concentrations of E2Eq ranged from below the QL (0.31 ng/L; 21 samples) to 7.2 ng/L. There were significant relationships between watershed density of AFOs and concentrations of DIN and E2Eq for all five sampling periods (Table 1). For both analytes, the proportion of variance explained by the relationship was greater during high flow (May; Table 1). As the watershed density of AFOs increased over 1 AFO/1000 acres, the frequency of DIN concentrations > 1000 μg/L and E2Eq concentrations > 1 ng/L generally increased (Figs. 2 and 3B). Concentrations of PO_4-P ranged from 1.5 to 1150 μg/L over the five sampling periods. The watershed density of AFOs predicted concentrations of PO_4-P for all sampling periods except March 2009 (Table 1), and the strength of these relationships and proportion of variance explained were less than those for DIN and E2Eq. Exceptionally high PO_4-P concentrations were measured at Muddy Creek in August 2008, March 2009, May 2009, and August 2009 (1.15×10^3, 349, 345, and 503 μg/L, respectively). These outliers were not included in final regression models of watershed density of AFOs and PO_4-P concentrations due to their

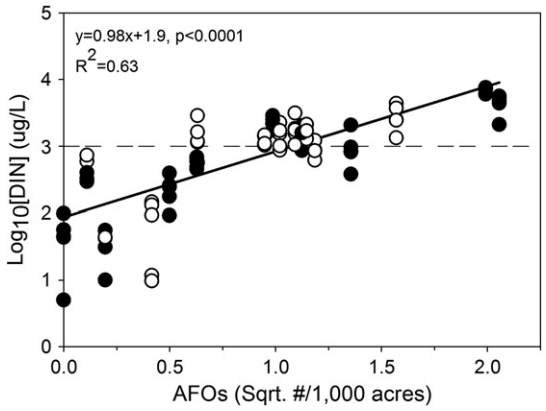

Fig. 2. Relationship between watershed density of AFOs and DIN concentrations. Data from five sampling periods are included. The control line represents 1000 μg/L. Filled symbols indicate sites with no upstream WWTPs and open symbols represent sites with at least one upstream WWTP. There was no significant effect of WWTP presence on DIN concentrations (ANCOVA, $p = 0.59$).

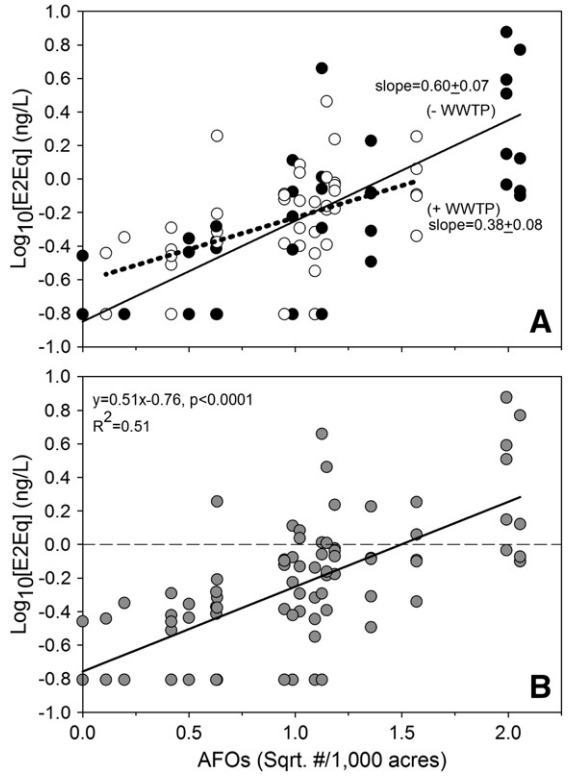

Fig. 3. Relationship between watershed density of AFOs and concentrations of E2Eq. Data from five sampling periods are included. A) Effects of WWTP presence on E2Eq concentrations. Filled symbols represent sites without upstream WWTPs and open symbols represent sites with WWTPs. Slopes were significantly different (ANCOVA, $p=0.02$), so a three-point evaluation was conducted and adjusted means were only significantly different at the lowest density of AFOs ($x=0.10$; $p=0.047$). B) Relationship between watershed density of AFOs and concentrations of E2Eq across all sampling periods. The control line represents 1 ng/L.

influence on the results (inflation of R^2 values and slopes) if included, and the multiple potential sources of PO_4-P at this site. The Muddy Creek watershed had the highest AFO density in this study, and the stream receives effluent from a poultry processing facility (unknown MGD), which warrants further investigation as a potential source of PO_4-P.

When compared to relationships with watershed percentages of pasture/hay and crops, relationships with watershed density of AFOs explained a greater proportion of variance in E2Eq, DIN, and PO_4-P concentrations for all sampling periods (Tables 1 and 2). Relationships between watershed percentage of developed land and the three analytes were not significant ($p\geq0.06$), with the exception of one significant relationship with PO_4-P ($R^2=0.25$, $p=0.04$) in August 2008. The combination of watershed percentages of pasture/hay and crops with watershed density of AFOs into an agricultural index (AI) did not result in explanation of a greater proportion of variance in analyte concentrations than watershed density of AFOs alone (Tables 1 and 2). Thus, watershed density of AFOs was the best predictor of concentrations of E2Eq, DIN, and PO_4-P.

Results of ANCOVA indicated no effect of sampling period ($p=0.40$) or WWTP presence ($p=0.59$) on DIN concentrations with regard to the overall relationship with watershed AFO densities (Table 3, Fig. 2). For E2Eq concentrations, ANCOVA indicated a significant effect of both sampling period ($p=0.02$) and WWTP presence ($p=0.03$), with regard to the overall relationship with watershed densities of AFOs. There was no significant interaction between sampling period and watershed density of AFOs ($p=0.56$), and the slopes were considered equal (Table 1). There was also no significant interaction between sampling period and WWTP presence ($p=0.60$), but there was a significant interaction between watershed densities of AFOs and WWTP presence ($p=0.02$). Separate models of the main effects were created in order to evaluate differences in adjusted means.

Because the equal slope hypothesis was rejected for the effect of WWTP presence on E2Eq concentrations (Fig. 3A), regression models and least-squares adjusted means were evaluated at three points ($x=0.10$, 0.94, and 1.87). This analysis indicated that the regression models were not equal at the lowest watershed AFO density ($p=0.047$), but were equal at the two higher AFO densities ($p\geq0.11$). Consequently, least-squares adjusted means were only significantly different at the lowest AFO density, where the adjusted mean E2Eq concentration was higher at sampling sites with at least one upstream WWTP (0.27 ± 0.05 ng/L) compared to sampling sites with no upstream WWTPs (0.16 ± 0.04 ng/L). However, these concentrations should be interpreted with caution as both are below the QL for E2Eq (0.31 ng/L) as a result of ½ QL substitutions for statistical analyses.

For sampling period, pairwise comparisons of least-squares adjusted means indicated that amongst the low flow sampling periods, adjusted mean E2Eq concentrations were significantly lower ($p\leq0.012$) in August 2008 than in March 2009 and August 2009 (Table 3). The only significant difference between high and low flow periods was higher adjusted mean E2Eq concentrations in March 2009 than in May 2009 ($p=0.035$, Table 3). As there were no consistent statistical differences between high and low flow conditions, all seasons were included in the final model of the relationship between watershed density of AFOs and E2Eq concentrations ($p<0.0001$, Fig. 3b). The effect of ½ QL substitution on the slope and intercept of this model was assessed using a Tobit analysis (Helsel, 2005). Results of this analysis indicated that relationship between AFOs on E2Eq concentrations was statistically significant ($p<0.0001$). The slope and intercept (±standard error) of the model derived from the Tobit analysis were 0.63 ± 0.07 and -0.93 ± 0.09, respectively. These were similar to the slope and intercept (±standard error) of the model derived from ½ QL substitution, which were 0.51 ± 0.05

Table 2
Proportions of variance (R^2) explained by linear regressions between analyte concentrations (y) and the independent (x) variables pasture/hay (PHay), cropland (Crop), and the agricultural index (AI).

y variable	E2Eq	E2Eq	E2Eq	DIN	DIN	DIN	PO_4-P	PO_4-P	PO_4-P
x variable	PHay	Crop	AI	PHay	Crop	AI	PHay	Crop	AI
May 2008	0.49†	0.42**	0.67††	0.71††	0.66††	0.76††	0.34**	0.23*	0.29*
Aug 2008	NS	NS	0.28*	0.38**	0.34**	0.45†	0.36**	0.40**	0.36**
Mar 2009	NS	0.26*	0.31*	0.50†	0.44**	0.50†	NS	NS	NS
May 2009	0.52†	0.49†	0.66††	0.62††	0.56†	0.71††	0.42**	0.50†	0.52†
Aug 2009	0.48**	0.42**	0.58†	0.48†	0.44**	0.52†	0.41**	0.45**	0.50†

NS: regression was not statistically significant ($p\geq0.05$).
* $p<0.05$.
** $p\leq0.01$.
† $p\leq0.001$.
†† $p\leq0.0001$: p-values of regression models.

Table 3
Mean analyte concentrations and loadings during each sampling period ± standard error, adjusted for relationships with watershed densities of AFOs (least-squares adjusted means), and ranges in measured discharge.

	Concentrations			Loadings[1]			Discharge[1]
	E2Eq	DIN	PO_4-P	E2Eq	DIN	PO_4-P	
	ng/L	μg/L	μg/L	mg/km^2/d	g/km^2/d	g/km^2/d	m^3/s
May 2008	0.48 ± 0.07^{abc}	794 ± 185	9.3 ± 2.2				
Aug 2008	0.39 ± 0.06^{a}	933 ± 217	15.8 ± 3.6				
Mar 2009	0.71 ± 0.10^{b}	490 ± 115	NS	0.14 ± 0.03^{a}	96 ± 30^{a}	NS	0.03–0.80
May 2009	0.44 ± 0.07^{ac}	741 ± 172	11.7 ± 2.7	0.31 ± 0.06^{b}	507 ± 159^{b}	7.9 ± 1.7^{a}	0.07–3.3
Aug 2009	0.66 ± 0.10^{bc}	708 ± 164	12.3 ± 2.8	0.09 ± 0.02^{a}	94 ± 29^{a}	1.5 ± 0.33^{b}	0.02–0.70

NS: relationship with AFOs was not statistically significant.
Superscript letters indicate statistically different concentrations or loadings between sampling periods.
[1] Loadings were not calculated in 2008 because discharge was not measured.

and −0.76 ± 0.06, respectively (Fig. 3B). Thus, the ½ QL substitution did not appear to have a substantial effect on the derived model.

For PO_4-P concentrations, ANCOVA did not include the March 2009 sampling period due to the lack of a significant univariate relationship with watershed density of AFOs. There was no effect of the other four sampling periods ($p = 0.42$) on PO_4-P concentrations (Table 3), but there was a significant effect of WWTP presence ($p = 0.0006$), when the relationship with AFOs was taken into account (Fig. 4), with no interaction between WWTP presence and watershed density of AFOs ($p = 0.26$). Least-squares adjusted mean PO_4-P concentrations were significantly higher ($p = 0.0008$) at sampling sites with at least one WWTP located upstream (16.6 ± 2.3 μg/L) compared to sampling sites with no upstream WWTPs (8.5 ± 1.2 μg/L). Comparison of unadjusted mean concentrations of PO_4-P in March 2009 also indicated significantly higher ($p = 0.01$) concentrations at sites with upstream WWTPs (12.0 ± 4.2 μg/L) compared to sites without upstream WWTPs (2.95 ± 1.0 μg/L). For sites with upstream WWTPs, the unadjusted mean March 2009 PO_4-P concentration was similar to the adjusted mean concentration during the other sampling periods (Table 3). However, for sites without upstream WWTPs, the unadjusted mean March 2009 PO_4-P concentration was lower than the adjusted mean concentration for the other sampling periods (Table 3).

Estimated loadings (normalized to watershed area) of E2Eq ranged from 0.01 to 1.8 mg/km^2/d and loadings of DIN ranged from 0.6 to 5841 g/km^2/d over the three sampling periods when discharge was measured (in 2009). Estimated loadings of PO_4-P ranged from 0.2 to 61 g/km^2/d over the three sampling periods, excluding loadings calculated from outlying concentrations measured in Muddy Creek (49, 377, 137 g/km^2/d in March, May, and August 2009, respectively), which were not included in statistical analyses. Similar to concentrations, there were significant relationships between watershed density of AFOs and loadings of DIN and E2Eq for all three sampling periods (Table 1), and watershed density of AFOs was the best predictor of loadings of all analytes relative to land cover variables (see Supplementary data). Watershed density of AFOs predicted loadings of PO_4-P in May and August 2009, but not in March (Table 1).

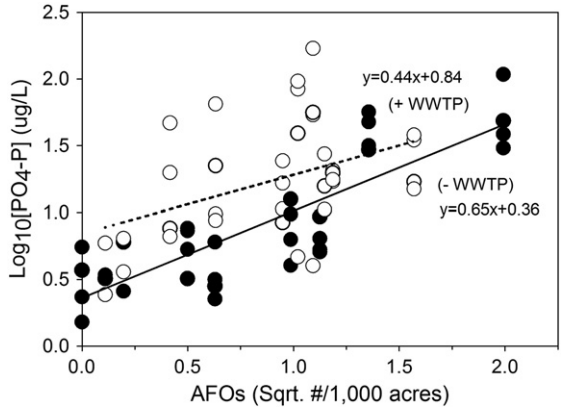

Fig. 4. Relationship between watershed densities of AFOs and PO_4-P concentrations. Data from four sampling periods are included (March 2009 excluded). Data from one site (Muddy Creek) were not included because of outlying concentrations. Filled symbols represent sites with no upstream WWTPs and open symbols represent sites with at least one upstream WWTP. There was a significant effect of WWTP presence (ANCOVA, $p = 0.0006$), indicated by the regression lines (+ or − WWTP). Slopes of regressions of the two categories of sites were not statistically different ($p = 0.26$), and the adjusted mean PO_4-P concentration was significantly higher for sites with at least one upstream WWTP compared to sites with no upstream WWTPs ($p = 0.0008$).

For loadings of all analytes (excluding PO_4-P in March), ANCOVA indicated a significant effect of sampling period ($p \leq 0.0002$) when the relationship with watershed density of AFOs was taken into account, with no interaction between variables ($p \geq 0.18$), including WWTP presence. Adjusted mean loadings of DIN and E2Eq were significantly higher in May ($p \leq 0.005$) than in March and August (Table 3). Adjusted mean loadings of DIN and E2Eq during March and August were not statistically different ($p > 0.093$). Adjusted mean loadings of PO_4-P were significantly higher in May than in August ($p < 0.0001$; Table 3). There was no significant effect of WWTP presence on loadings of PO_4-P (excluding March), E2Eq, and DIN ($p = 0.064$, 0.10, and 0.41, respectively). Unadjusted mean loadings of PO_4-P in March were significantly higher ($p = 0.011$) at sites with at least one upstream WWTP (2.6 g/km^2/d) compared to sites with no upstream WWTPs (0.56 g/km^2/d).

Concentrations of DIN were correlated with concentrations of E2Eq ($r = 0.56$, $p < 0.0001$) and PO_4-P ($r = 0.51$, $p < 0.0001$), and there was a weaker correlation between concentrations of PO_4-P and E2Eq ($r = 0.35$, $p = 0.001$). Loadings of all three analytes were correlated, including DIN and E2Eq ($r = 0.69$, $p < 0.0001$), DIN and PO_4-P ($r = 0.63$, $p < 0.0001$), and PO_4-P and E2Eq ($r = 0.63$, $p < 0.0001$).

4. Discussion

4.1. Concentrations and sources of DIN and estrogenic activity

Results of this study suggest that as densities of AFOs increase in watersheds of Shenandoah River tributaries, the amount of manure applied in the local area increases, which directly affects in-stream concentrations of nutrients and estrogenic activity. Watershed densities of AFOs had the strongest relationships with in-stream DIN concentrations during all sampling periods. A similar relationship between NO_3-N concentrations and AFO densities was described by Weldon and Hornbuckle (2006) for Iowa watersheds that were larger (area > 500 km^2) than watersheds in the current study (38–334 km^2). The similar effect of watershed AFO density on dissolved N concentrations between two different ecoregions and size classes of streams supports previous general hypotheses that the risk of nonpoint source nutrient pollution is greater for streams in regions with high densities of AFOs (Carpenter et al., 1998;

Kellog et al., 2000; Mallin and Cahoon, 2003). In this study, over 50% of the water samples had DIN concentrations >1000 μg/L and these samples were primarily collected from tributaries with watershed AFO densities >1 AFO/1000 acres. These concentrations indicate potential risk for local eutrophication; proliferation of benthic algae has been documented at DIN and total N concentrations less than 500 μg/L (Biggs, 2000; Dodds et al., 1997; Miltner, 2010). In addition, the ability of streams to remove NO_3-N through biotic uptake decreases rapidly between 150 and 1500 μg/L (Mullholland et al., 2008). The strength of the relationship between DIN concentrations at the watershed outlet and watershed AFO density suggests that the studied Shenandoah River tributaries are exporting a large proportion of catchment-derived DIN. Downstream transport of DIN to the Shenandoah River and beyond is of concern due to the imperiled condition of Chesapeake Bay, where phytoplankton growth is N-limited in higher salinity areas and during the summer (Boesch et al., 2001). Mullholland et al. (2008) highlighted the importance of controlling nitrogen export by small streams in order to prevent downstream problems with eutrophication and hypoxia.

Relationships between watershed density of AFOs and in-stream estrogenic activity were similar to those for DIN. Estrogenic activity in Shenandoah River tributaries with high watershed densities of AFOs may present a risk to aquatic biota. Using a compilation of studies on endocrine disruption in fish, Young et al. (2004) derived 1 ng/L E2Eq as the predicted no-effect concentration of total estrogens on fish reproduction. In the current study, 18 samples from 10 sites had concentrations >1 ng/L E2Eq, and the majority of these sites had watershed AFO densities >1 AFO/1000 acres. Although land-applied manure from AFOs is a diffuse source of estrogens, the effect of watershed application rates on estrogenicity in streams appears to be cumulative. The range in estrogenic activity observed during this study was similar to estrogen concentrations and total activity measured in streams with more concentrated sources, including: streams draining fields receiving waste from poultry AFOs (up to 5 ng/L E2; Shore et al., 1995), streams adjacent to pastures receiving waste from dairy and beef AFOs with or without grazing ewes (up to 9.2 ng/L E2Eq; Matthiessen et al., 2006), and rangeland creeks accessed by grazing cattle (up to 10.6 ng/L E2Eq assuming activity of 17α-estradiol = 0.05*E2; Kolodziej and Sedlak, 2007). A similar range in estrogenic activity (up to 7 ng/L E2Eq) has also been measured in treated WWTP effluent (Salste et al., 2007) and in rivers receiving effluent from WWTPs (Vermeirssen et al., 2005). However, there was no consistent measurable effect of WWTPs on estrogenic activity in this study. The apparent effect of WWTPs on estrogenic activity at a low watershed density of AFOs was due to only three samples. At higher AFO densities, estrogenic activity was nearly identical between sites with and without WWTPs. The effect of watershed density of AFOs appeared to outweigh any effect of WWTPs on in-stream estrogenic activity in these tributaries, which may be due to the low effluent volume generated by the WWTPs.

Watershed density of AFOs explained the greatest proportion of variance in concentrations of both DIN and E2Eq during high flow conditions (May). Manure is applied almost year-round in Virginia (except mid-winter), but application rates are generally greatest in late spring/early summer (Marsh et al., 2009). The combination of high manure application rates and heavy rainfall during late spring may facilitate the transport of DIN and estrogens/estrogenic compounds to surface waters. Potential mechanisms of transport of contaminants in land-applied manure to surface waters include both runoff from manure-treated fields (Finlay-Moore et al., 2000; Jenkins et al., 2006; Mishra et al., 2006; Nichols et al., 1997; Sistani et al., 2010; Soupir et al., 2006), and leaching into soil water and subsequent transfer through interflow (Daliparthy et al., 1995; Herman and Mills, 2003; Hyer et al., 2001; Kjaer et al., 2007). Higher solubility of DIN compared to compounds contributing to estrogenic activity may account for the stronger relationships between DIN and watershed density of AFOs during all sampling periods. For both analytes, the lack of significantly higher concentrations during high flow conditions is likely due to the watershed-scale sampling design; runoff and interflow from areas with and without AFOs are combined in stream water sampled at the watershed outlet.

During summer low flow conditions (August) and the unusual late winter low flow condition in 2009, significant relationships between watershed densities of AFOs and in-stream concentrations of DIN and estrogens/estrogenic compounds and were maintained. This suggests that the presence and dynamics of estrogens and nutrients in groundwater require further study in the Shenandoah River watershed. Groundwater contamination may result from the spatial correlation between karst features and high densities of AFOs in the valley region of the watershed. The generally consistent DIN concentrations and estrogenic activity between high and low flow conditions are biologically relevant; organisms inhabiting streams draining watersheds with high densities of AFOs may experience continuous exposure to relatively high concentrations.

4.2. Concentrations and sources of PO_4-P

Relative to the other analytes, relationships between watershed density of AFOs and PO_4-P concentrations were weaker and there was no consistent change in the proportion of variance explained during high flow conditions. These differences may be due to the tendency of phosphates to sorb to particles, resulting in lower solubility (Mueller and Spahr, 2006), and/or rapid biological uptake of PO_4-P (Dodds and Welch, 2000). However, linear relationships between PO_4-P and watershed density of AFOs during both spring high and summer low flow sampling periods suggest that P is moving from land applied manure into surface water and possibly groundwater. Despite the tendency for sorption, a large proportion of the P lost from manure-treated fields can be in the dissolved form (Sistani et al., 2010; Withers and Jarvie, 2008).

Orthophosphate was the only analyte with a significant relationship to WWTPs. Streams receiving WWTP effluent had higher PO_4-P concentrations than those streams without WWTPs during all sampling periods. Elevated concentrations of PO_4-P downstream of WWTPs have been previously observed (Mueller and Spahr, 2006), due to high P concentrations in the effluent and the effluent's highly soluble nature (Withers and Jarvie, 2008). The combined effect of watershed AFO density and WWTPs on PO_4-P concentrations has important implications for management of the nutrient that typically limits primary production in freshwater environments. Upgrading the WWTPs to a level of treatment that includes nutrient removal would limit a direct source of PO_4-P to the studied tributaries, but streams would still receive PO_4-P from manure-treated fields. In addition, a significant portion of P may be sorbed to suspended particles or sediment in streams (Withers and Jarvie, 2008), particularly in agricultural areas (Klotz, 1985; Palmer-Felgate et al., 2009). Rapid mineralization of the sorbed P can lead to eutrophic conditions (Dodds and Welch, 2000). The P-based risk of eutrophication in Shenandoah River tributaries was likely underestimated by the measured PO_4-P concentrations, and minimization of this risk would require further understanding of in-stream P cycling.

4.3. Loadings of all analytes

Loadings of all analytes had linear relationships to watershed density of AFOs and other land uses similar to concentrations, but the proportion of variance in analyte loadings explained by these relationships was generally less than that of analyte concentrations. This may be due to effect of stream discharge, which is unrelated to land use, on loading calculations. Weldon and Hornbuckle (2006) saw a similar pattern when comparing relationships of NO_3-N concentrations and loadings with watershed density of AFOs. Unlike concentrations, there were significant seasonal differences in loadings of

all analytes. Higher loadings during the late spring sampling period indicate that larger amounts of nutrients and estrogens/estrogenic compounds are entering and being exported by Shenandoah River tributaries during high flow conditions. This increases the risk to receiving waters in terms of aquatic organism exposure to endocrine-disrupting compounds and delivery of excess nutrients. Fish kills in the Shenandoah River have generally occurred in late spring (Blazer et al., 2010), and while far from causative, there is an apparent temporal link between the fish kills and higher loadings of landscape-derived contaminants. The moderate correlations between concentrations of DIN, PO_4-P, and estrogenic activity are likely due to differences in in-stream processing of the compounds including: solubility, potential for biological uptake (nutrients), and degradation (estrogens). However, the stronger correlations between loadings of DIN, PO_4-P, and estrogens/estrogenic compounds suggest that the amounts of these contaminants entering Shenandoah River tributaries are related to each other and to landscape sources.

4.4. Additional sources of variability

Although the results of this study suggest that manure from AFOs is a primary contributor of nutrients and estrogens/estrogenic compounds to Shenandoah River tributaries, there are recognized limitations within the dataset. First, all potential sources of these contaminants were not included in the study design. For example, septic fields are a potential source of nutrients and estrogens/estrogenic compounds to groundwater and the use of inorganic N fertilizers on crops is a potential source of DIN (Lindsey et al., 2003; Swartz et al., 2006). The use of both inorganic fertilizers and manure on crops may result in greater transport of DIN to streams than either source alone. Septic fields and inorganic fertilizer on cropland did not appear to be overly influential to in-stream analyte concentrations, given the analytes' weaker linear relationships with watershed percentages of cropland compared to watershed density of AFOs, and generally insignificant relationships with watershed percentage of developed land. However, confirmation of the relative influence of these sources would require knowledge of locations and usage within each watershed. A second limitation is that the SPE methodology and use of the BLYES were designed to capture total estrogenicity in Shenandoah River tributaries, as an indication of potential endocrine-disrupting effects on aquatic organisms. The relative contribution of steroid estrogens, their conjugates, phyto- and myco-estrogens, as well as synthetic xenoestrogens to the measured estrogenic activity would require analysis by chromatography and mass spectrometry, which was beyond the scope of this study. The presence of estrogen receptor antagonists also requires further study as the BLYES indicates the presence of compounds that can bind to the estrogen receptor, but does not distinguish between estrogen agonists and antagonists (Sanseverino et al., 2005). A third limitation is that the spatial overlap of different types of AFOs in the Shenandoah River watershed complicates the association of nutrients and estrogenic activity with specific animal sources. Poultry houses are the numerically dominant type of AFO, but most watersheds with high densities of poultry houses also have high densities of dairy AFOs and/or beef cattle. The relative impact of one particular source of manure compared to the synergistic effect of all types of manure within the watersheds of Shenandoah River tributaries requires further study before more effective manure management practices can be implemented.

5. Conclusions

Results of this study suggest that increasing watershed densities of AFOs results in increasing concentrations of nutrients and compounds with endocrine-disrupting potential in agricultural streams. In watersheds with high agricultural intensity and low development, the influence of AFOs on in-stream concentrations of DIN and estrogenic activity appears to outweigh the effects of small WWTPs. Minimizing the cumulative effect of AFOs on water quality may require implementation of watershed-scale manure management plans for nutrients. The apparent cumulative effects of AFOs on estrogenicity suggest that endocrine-disrupting potential in agricultural streams should be monitored at the landscape scale. Biologically-based screening methods such as the BLYES may offer a cost-effective approach to monitor estrogenicity on a broad spatial scale and identify at-risk areas for further assessment of the potential effects of estrogenic compounds on aquatic organisms in agricultural streams.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.scitotenv.2011.10.017.

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