

Phytoestrogens and Mycotoxins in Iowa Streams: An Examination of Underinvestigated Compounds in Agricultural Basins

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This study provides the first broad-scale investigation on the spatial and temporal occurrence of phytoestrogens and mycotoxins in streams in the United States. Fifteen stream sites across Iowa were sampled five times throughout the 2008 growing season to capture a range of climatic and crop-growth conditions. Basin size upstream from sampling sites ranged from 7 km² to >836,000 km². Atrazine (herbicide) also was measured in all samples as a frame-of-reference agriculturally derived contaminant. Target compounds were frequently detected in stream samples: atrazine (100%), formononetin (80%), equol (45%), deoxynivalenol (43%), daidzein (32%), biochanin A (23%), zearalenone (13%), and genistein (11%). The nearly ubiquitous detection of formononetin (isoflavone) suggests a widespread agricultural source, as one would expect with the intense row crop and livestock production present across Iowa. Conversely, the less spatially widespread detections of deoxynivalenol (mycotoxin) suggest a more variable source due to the required combination of proper host and proper temperature and moisture conditions necessary to promote *Fusarium* spp. infections. Although atrazine concentrations commonly exceeded 100 ng L⁻¹ (42/75 measurements), only deoxynivalenol (6/56 measurements) had concentrations that occasionally exceeded this level. Temporal patterns in concentrations varied substantially between atrazine, formononetin, and deoxynivalenol, as one would expect for contaminants with different source inputs and processes of formation and degradation. The greatest phytoestrogen and mycotoxin concentrations were observed during spring snowmelt conditions. Phytoestrogens and mycotoxins were detected at all sampling sites regardless of basin size. The ecotoxicological effects from long-term, low-level exposures to phytoestrogens and mycotoxins or complex chemicals mixtures including these compounds that commonly take place in surface water are poorly understood and have yet to be systematically investigated in environmental studies.

PHYTOESTROGENS AND MYCOTOXINS are naturally occurring compounds that are derived from a wide variety of plant and fungal species. Primary sources of phytoestrogens include legumes such as clover (*Trifolium* spp.) and soybean [*Glycine max* (Merr.) L.] (Sivesind and Seguin, 2005; Morrison et al., 2008), some fraction of which may be released from agricultural crops and fields into the environment (Erbs et al., 2007; Hoerger et al., 2009). Additional pathways to the environment for phytoestrogens can be from human and animal excretion after plant consumption (Burnison et al., 2003; Heinonen et al., 2004; Ferrer et al., 2009; Kang and Price, 2009; Bester et al., 2010). Deleterious effects have been observed to wild and domestic animals consuming forage containing phytoestrogens (Leopold et al., 1975; Adams, 1995). Beneficial properties of phytoestrogens, however, have also been documented (Messina, 1995; Knight and Eden, 1996; Choo et al., 2002). Mycotoxins are metabolites of fungal species that can grow on a wide variety of crops, including wheat (*Triticum* spp.) and corn (*Zea mays* L.), with *Fusarium* spp. being among the most important toxigenic fungi (Goswami and Kistler, 2004). In addition, some phytoestrogens and mycotoxins are known to be estrogenic (Breinholt and Larsen 1998; Coldham et al., 1997; Knudsen and Pottinger, 1999; Liu et al., 2010; Matthews et al., 2000; Poulin et al., 1997) and as such could be important compounds contributing to the total estrogenicity in the environment. Although extensive research has been conducted on the production of phytoestrogens and mycotoxins and their occurrence in agricultural products (Lundh, 1995; Pittet, 1998), little has been done to determine their environmental distribution (Hartmann et al., 2008a; Hartmann et al., 2008b; Bucheli et al., 2008; Wettstein and Bucheli, 2010). To our knowledge, no such broad-scale research on these compounds has been conducted to date in the United States.

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Abbreviations: ATZ, atrazine; BIO, biochanin A; COU, coumestrol; DAI, daidzein; DAR, deethylatrazine to atrazine ratio; DEA, deethylatrazine; DON, deoxynivalenol; FOR, formononetin; GEN, genistein; ZON, zearalenone.

The midwestern United States is an area of intensive agricultural activity in terms of crop and livestock production, with Iowa (Fig. 1) being in the center of such activity. There were roughly 5.69 million ha of corn, 3.48 million ha of soybean, 0.46 million ha of forage crops (e.g., alfalfa, grass), 19.3 million swine, 3.98 million cattle, and 69.2 million poultry produced in Iowa in 2007 (USDA National Agricultural Statistics Service, 2009a). Thus, Iowa was an ideal location to test the hypothesis that phytoestrogens and mycotoxins are present in streams having extensive agricultural activities such as crop and livestock production.

The purpose of this report is to describe the occurrence of five isoflavones, one coumestan, and two mycotoxins in selected rivers and streams across Iowa during the 2008 growing season. This study provides the first broad-scale investigation on the spatial and temporal occurrence of such natural contaminants in streams conducted in the United States.

Materials and Methods

Sampling Sites

To determine the prevalence of phytoestrogens and mycotoxins in agricultural-affected rivers and streams, a network of 15 sampling sites across Iowa was selected to provide a range of basin sizes and geographic distribution across the state (Table 1; Fig. 1). These sites include 13 interior stream basins and two sites on the large border rivers of Iowa (Missouri and Mississippi Rivers) that also include extensive basin areas outside of Iowa. This stream network drains about 90% of Iowa. These sampling sites were selected because they were located at a U.S. Geological Survey (USGS) streamgage in order that continuous streamflow could be provided and were being frequently sampled as part of other ongoing water quality investigations (e.g., Meyer et al., 2007). All sites were sampled five times during the 2008 crop growing season: March (pregrowing season), June (early growing season), July/August (mid-growing season), September (late growing season), and October (harvest) (Fig. 2). Sites were generally sampled within a 2-wk window during each collection period. All samples were collected by USGS personnel using standard depth and width integrating techniques (Shelton, 1994) to obtain a representative stream sample. Water samples were filtered through a 0.7- μ m glass-fiber filter into amber baked-glass bottles and immediately chilled and shipped to the laboratory.

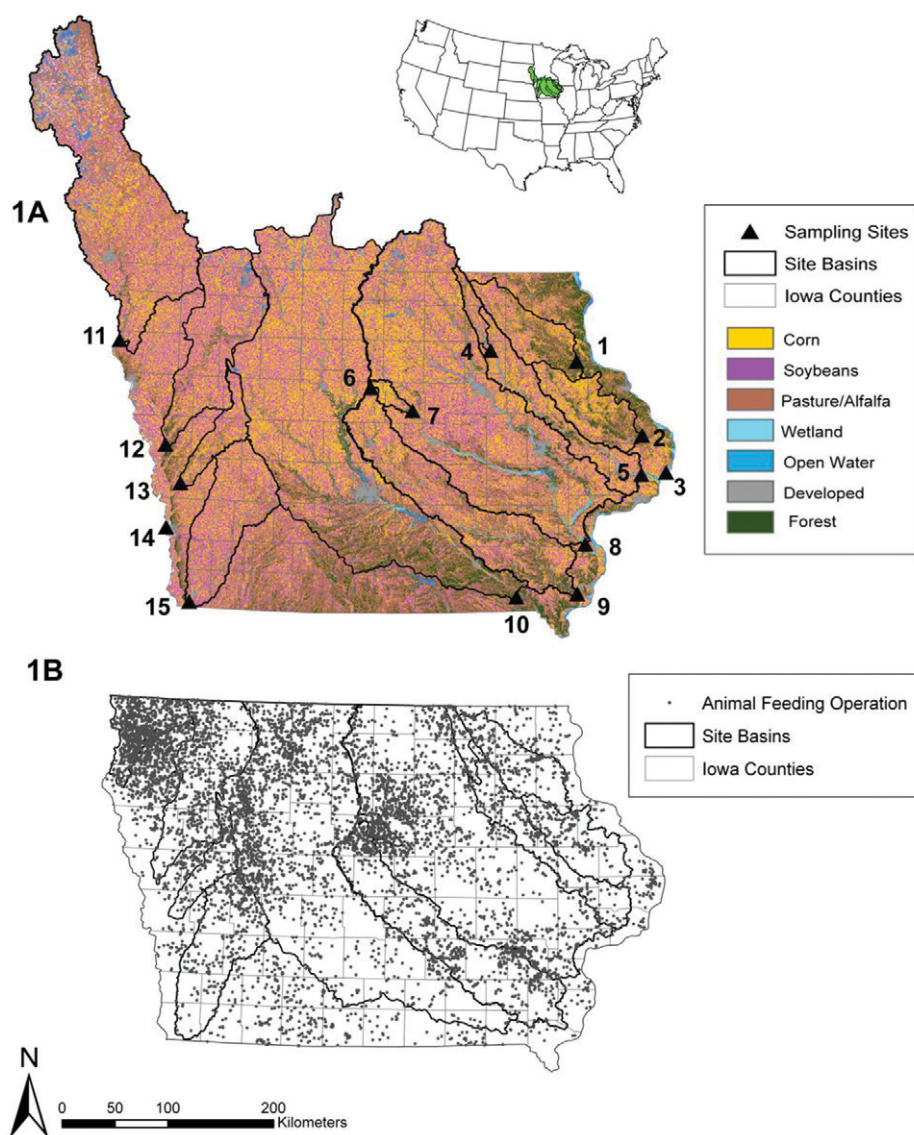


Fig. 1. (A) Land use (USDA National Agricultural Statistics Service, 2009b) and (B) permitted animal feeding operations (Iowa Geological Survey, 2009) in Iowa. Stream sites where water samples were collected for analysis in 2008 are designated with black triangles. The number adjacent to this map symbol denotes the corresponding map number that is cross-referenced in Table 1.

Chemical Analysis

All samples were analyzed for the isoflavones biochanin A (BIO), daidzein (DAI), equol, formononetin (FOR), and genistein (GEN); the coumestan coumestrol (COU); the mycotoxins deoxynivalenol (DON) and zearalenone (ZON); the herbicide atrazine (ATZ); and a major ATZ degradate deethyl-atrazine (DEA) (Table 2). Biochanin A and FOR represent the main isoflavones in important fodder crops such as red clover (Sivesind and Seguin, 2005). Daidzein and GEN are not only the primary metabolites of BIO and FOR but are also derived from soybean and other legumes (Morrison et al., 2008). Equol is a metabolite of DAI that is not produced *in planta* but rather is metabolized in the human gut and in the rumen of domestic animals (Lundh, 1995; Heinonen et al., 2004). Coumestrol was selected because it is the phytoestrogen exhibiting the highest relative estrogenic potency (Coldham et al., 1997; Matthews et al., 2000; Bovee et al., 2004). Zearalenone was selected because of its pronounced estrogenicity that is

Table 1. Network of stream sampling sites in Iowa and streamflow encountered during each sampling period.†

Map no.‡	Station ID	Stream name	Drainage area	Streamflow				
				Mar.	June	July/Aug.	Sept.	Oct.
			km ²	m ³ s ⁻¹				
1	05412500	Turkey River at Garber	4,002	36.5	884	25.0	16.8	11.7
2	05418600	Maquoketa River at Spragueville	4,227	37.1	796	45.6	28.0	21.6
3	05420500	Mississippi River at Clinton	221,704	1210	2040	858	685	830
4	05420680	Wapsipinicon River near Tripoli	896	10.8	442	2.46	1.02	1.02
5	05422000	Wapsipinicon River near De Witt	6,035	60.3	303	34.6	15.6	14.1
6	05451070	South Fork Iowa River Headwaters near Blairsburg	7	0.2	1.39	0.01	0.01	0.02
7	05451210	South Fork Iowa River northeast of New Providence	580	36.2	163	2.8	0.42	0.48
8	05465500	Iowa River at Wapello	32,375	558	935	476	198	123
9	05474000	Skunk River at Augusta	11,145	175	640	66.3	34.0	48.4
10	05490500	Des Moines River at Keosauqua	36,358	513	3000	561	70.5	274
11	06485500	Big Sioux River at Akron	21,818	30.9	337	40.2	14.7	76.5
12	06607500	Little Sioux River near Turin	9,132	68.0	351	60.9	20.3	38.8
13	06609500	Boyer River at Logan	2,256	45.0	391	22.0	9.91	17.2
14	06610000	Missouri River at Omaha	836,052	632	1140	818	821	705
15	06810000	Nishnabotna River at Hamburg	7,268	57.8	651	110	26.4	41.4
			median flow	57.8	640	45.6	20.3	38.8

† Data from USGS National Water Information System (2009).

‡ See Fig. 1.

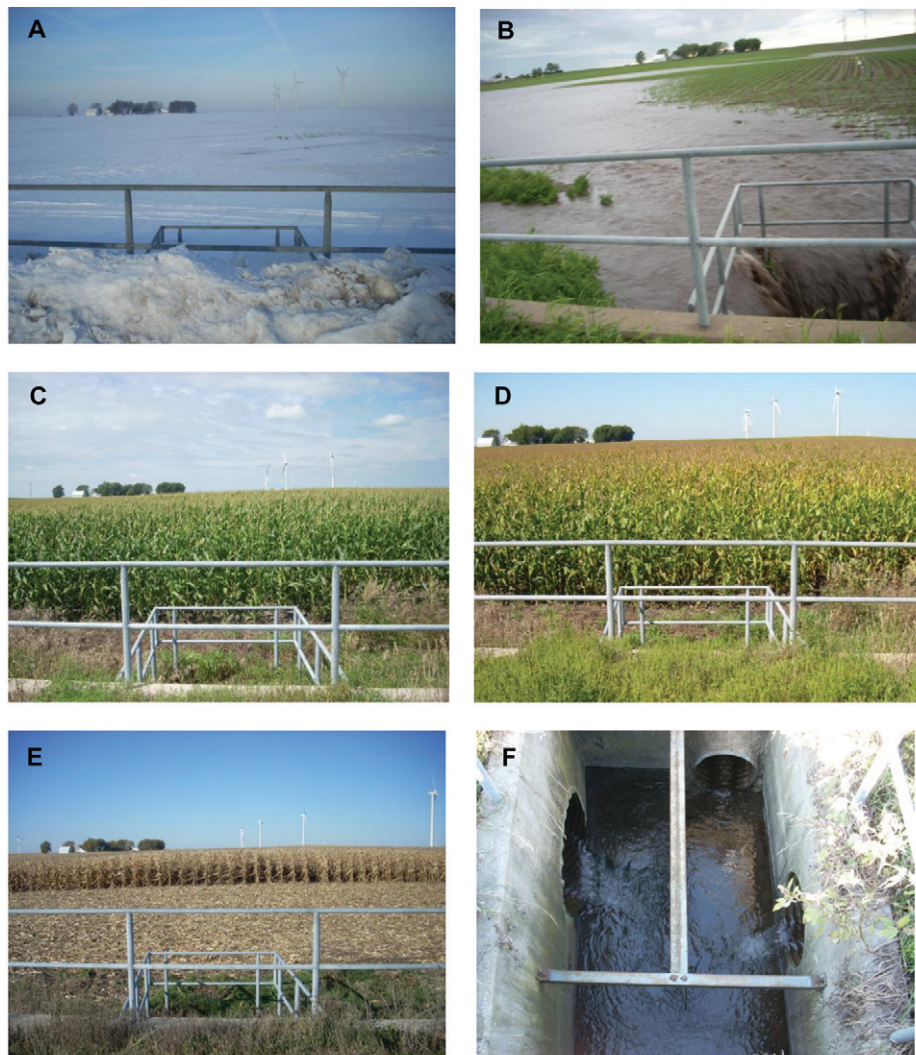
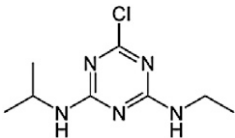
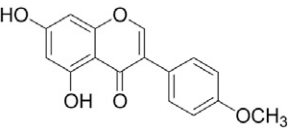
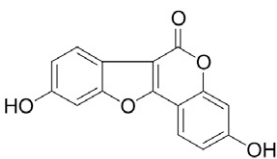
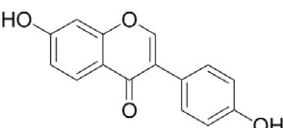
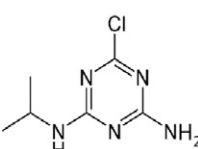
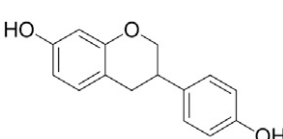
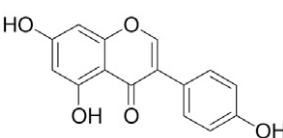
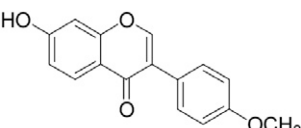
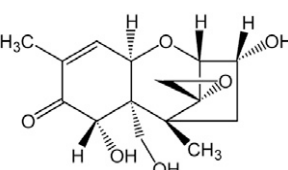
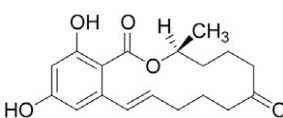


Fig. 2. Time series of photographs taken at South Fork Iowa River at headwaters (05451070) showing the general climatic, hydrologic, and crop growth conditions encountered during the five sampling rounds in 2008. (A) March: pregrowing season, snowmelt conditions. (B) June: early growing season, intense flooding conditions. (C) July/August: mid-growing season, normal flow conditions. (D) September: late-growing season, dry conditions. (E) October: harvest, increased flow conditions. (F) view of headwaters during October. All photos taken by Kate Segreto (U.S. Geological Survey).

Table 2. Targeted phytoestrogens, mycotoxins, and herbicide compounds investigated for this study.

Compound	CAS number	Molecular structure	Molecular formula	Log K_{ow}	Origin
Atrazine (ATZ) 2-chloro-4-ethylamino-6-isopropylamino-s-triazine	1912-24-9		$C_8H_{14}ClN_5$	2.6†	herbicide
Biochanin A (BIO) 5,7-dihydroxy-3-(4-methoxyphenyl)chromen-4-one	491-80-5		$C_{16}H_{12}O_5$	3.4‡	isoflavone (e.g. red clover)
Coumestrol (COU) 3,9-dihydroxy-6-benzofurano[3,2-c]chromenone	479-13-0		$C_{15}H_8O_5$	1.6‡	coumestan (e.g. alfalfa, soybean, spinach)
Daidzein (DAI) 7-hydroxy-3-(4-hydroxyphenyl)chromen-4-one	486-66-8		$C_{15}H_{10}O_4$	2.6‡, 2.5§	isoflavone (e.g. soybean)
Deethylatrazine (DEA) 2-amino-4-chloro-6-(isopropylamino)-s-triazine	6190-65-4		$C_6H_{10}ClN_5$	2.5†	Atrazine degradate
Equol (3S)-3-(4-hydroxyphenyl)-7-chromanol	94105-909-5		$C_{15}H_{14}O_3$	3.7‡, 3.2§	isoflavone (metabolite of digestion process)
Genistein (GEN) 5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one	446-72-0		$C_{15}H_{10}O_5$	2.8‡, 3.0§	isoflavone (e.g. soybean)
Formononetin (FOR) 7-hydroxy-3-(4-methoxyphenyl)chromen-4-one	485-72-3		$C_{16}H_{12}O_4$	3.1‡	isoflavone (e.g. red clover)
Deoxynivalenol (DON) (3a,7a)-3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one	51481-10-8		$C_{15}H_{20}O_6$	-0.7‡	mycotoxin (<i>Fusarium graminearum</i>)
Zearalenone (ZON) (3S,11E)-14,16-dihydroxy-3-methyl-3,4,5,6,9,10-hexahydro-1H-2-benzoxacyclotetradecine-1,7(8H)-dione	17924-92-4		$C_{18}H_{22}O_5$	3.6‡	mycotoxin (<i>Fusarium graminearum</i>)

† Experimental (Finizio et al., 1991). Both values are in close agreement with those recommended by the FOOTPRINT Pesticide Properties Database (<http://sitem.herts.ac.uk/aeru/footprint/en/>).

‡ Estimated, calculation with KOWWIN v1.67a (Sept. 2008) 2000 U.S. Environmental Protection Agency.

§ Experimental, pH = 7.4 (Rothwell et al., 2005).

dramatically above those of phytoestrogens (Coldham et al., 1997; Matthews et al., 2000). Deoxynivalenol was selected because previous research has shown it to be frequently present in Swiss streams (Bucheli et al., 2008). Atrazine and DEA were selected as reference agricultural contaminants because these compounds have been found to be essentially ubiquitous in streams across the Midwest (Battaglin et al., 2005).

The phytoestrogens (BIO, COU, DAI, equol, FOR, GEN) and mycotoxins (DON, ZON) were solid-phase extracted from aqueous samples, followed by separation with liquid chromatography-tandem mass spectrometry. The internal standards $^{13}\text{C}_3$ -FOR, $^{13}\text{C}_3$ -DAI, $^{13}\text{C}_3$ -equol, $^{13}\text{C}_3$ -BIO, $^{13}\text{C}_3$ -GEN (STANDIL; St. Andrews, Fife, Scotland), d_6 -ZON (internally produced by base catalyzed hydrogen-deuterium exchange on native ZON) (Miles et al., 1996), and $^{13}\text{C}_{15}$ -DON (Biopure Referenzsubstanzen GmbH, Tulln, Austria) were added together as 50 μL of a 2 ng μL^{-1} methanol solution into the filtered 1-L water samples before extraction of the phytoestrogens and mycotoxins with OASIS HLB (6 mL, 200 mg) SPE cartridges (Waters Corp., Milford, MA). Because no internal standard was available for COU, quantification was done with $^{13}\text{C}_3$ -GEN. These internal standards largely compensated for any losses during extraction or caused by ion suppression. The SPE cartridges were conditioned with 5 mL of methanol and 5 mL Milli-Q water, consecutively. Water samples were drawn by vacuum through the cartridges at a flow rate of 5 to 10 mL min^{-1} . The cartridges were subsequently washed with 5 mL of Milli-Q water and dried by vacuum. Finally, the analytes were eluted with 10 mL of methanol into conical microreaction vials and evaporated to dryness using a gentle nitrogen gas stream. The dried extracts were reconstituted in 300 μL of Milli-Q water/acetonitrile (80:20, v/v) and transferred into amber glass vials. The samples were stored at 4°C and analyzed within 48 h. Liquid chromatography-tandem mass spectrometry was performed on a Varian 1200L LC-MS instrument (Varian, Inc., Walnut Creek, CA). The phytoestrogens were separated on a XTerra MS C18 column (2.1 mm $\times$ 100 mm, 3.5 μm) (Waters Corp., Milford, MA) connected to a XTerra MC C18 guard column (2.1 by 20 mm, 3.5 μm) (Waters Corp.). The mycotoxins were separated on a Polaris C18-A column (2.0 by 50 mm, 3 μm) (Varian, Inc.). The analytical methods for measuring phytoestrogens (Erbs et al., 2007), mycotoxins (Hartmann et al., 2007; Bucheli et al., 2008), and ATZ and DEA (Sandstrom et al., 2001) have been described previously. All samples were shipped within 3 d after collection to the USGS Organic Geochemical Research Laboratory, Lawrence, Kansas, for extraction. Samples were placed in chilled storage when immediate shipment to the laboratory was not possible (e.g., Friday collection). The solid-phase extraction cartridges were then shipped to Agroscope Reckenholz-Tänikon Research Station, Switzerland, where the elution from cartridges and the analysis of the target compounds took place. No mycotoxin data for all samples from the June collection and four samples from the July/August collection were available due to improper sample extraction.

Long-term data from 1230 surface-water matrix spikes showed a consistent underestimation in actual DEA concentrations, with median spike recoveries at 47% (Martin et al., 2009). Thus, DEA data were adjusted for modeled recovery

in matrix spikes (Martin et al., 2009) to provide a more realistic estimate of the actual concentrations present at the time of sampling.

Quality Assurance Protocol

Analytical quality-control parameters (ion suppression, absolute and relative recovery, method detection limits, and instrument linearity) for phytoestrogens and mycotoxins in different environmental aqueous samples were determined previously (Erbs et al., 2007; Hartmann et al., 2007; Bucheli et al., 2008). The limits of detection of the investigated compounds in river water were as follows: BIO (0.5 ng L^{-1}), COU (2.8 ng L^{-1}), DAI (1.8 ng L^{-1}), equol (0.6 ng L^{-1}), FOR (0.8 ng L^{-1}), GEN (2.2 ng L^{-1}), DON (1.5 ng L^{-1}), ZON (0.7 ng L^{-1}), ATZ (7 ng L^{-1}), and DEA (14 ng L^{-1}).

A field quality assurance protocol was used to determine the effect, if any, of field equipment and procedures on the concentrations of the phytoestrogens and mycotoxins in water samples. Field blanks, made from laboratory-grade organic free water, were submitted four times during this study and analyzed for all target compounds. Field blanks were subject to the same sample processing, handling, and equipment as the stream samples. No detections of phytoestrogens or mycotoxins were measured in any of the field blanks collected.

Field duplicates were collected five times during this study to quantify reproducibility of the data. Field duplicates were water samples collected along with the regular environmental sample and processed as if they had been obtained at a unique site. The presence or absence of the target compounds was confirmed in 37 of the 40 determinations. In three samples, GEN was detected in only one of the regular/duplicate pairs. In these cases, the unconfirmed detections were at the lower end of the reporting level for GEN. For the 15 regular/duplicate pairs having a confirmed presence of a phytoestrogen or mycotoxin, the relative percent difference ranged from 0 to 23 (median relative percent difference = 5).

Statistical Methods and Basin Characteristics

Nonparametric statistical techniques were used to determine statistical significance for this study. These methods were appropriate because the data did not exhibit normal distributions and because of the presence of left-censored water-quality data (concentrations less than analytical reporting levels). The Kruskal-Wallis test (Helsel and Hirsch, 1992) was used to test for spatial differences in the medians of two or more groups. Spearman's rank correlation was used to test the monotonic relation between two continuous variables. A significance level of 0.05 was used for all statistical tests in this study.

Land-use classification (Fig. 1A) was derived from satellite imagery (56-m resolution) collected during 2009 (USDA National Agricultural Statistics Service, 2009b). For determination of statistical relations to phytoestrogens and mycotoxins, the land-use data were normalized by dividing the area of each land use class by the basin area. Because of the large basin areas, land use information was not compiled for the Missouri River at Omaha and the Mississippi River at Clinton (Table 1). Data on animal feeding operations (Fig. 1B) were derived from located and permitted confinements and feedlots as of 2007 (Iowa Geological Survey, 2009). Similar information on

animal feeding operations was not available for areas outside of Iowa. Thus, for determining statistical relations to phytoestrogen and mycotoxins, livestock information (e.g., total animal units, live weight, and number of operations) was summed by basin and divided by basin area within Iowa to generate various livestock density factors.

Results and Discussion

The five sampling rounds captured a range of climatic (winter, spring, summer, fall), hydrologic (low flow to high flow), and crop growth conditions (preplanting through harvest) (Fig. 2; Table 1). This design aspect was critical to accomplishing the goal of defining the occurrence of phytoestrogens and mycotoxins in streams in an intensely farmed area of row crop and livestock agriculture. An example of the flow conditions encountered during this study is provided in Fig. 3. In particular, the March sampling captured runoff conditions (Fig. 3) associated with the melting of an above normal snowpack (Buchmiller and Eash, 2010; Holmes et al., 2010). In addition, the June sampling captured record flooding conditions (Fig. 2 and 3) that persisted across much of Iowa (Buchmiller and Eash, 2010; Holmes et al., 2010), with many of the sampling sites approaching or exceeding the 0.2% flood probability.

Atrazine

As a frame of reference for phytoestrogens and mycotoxins, ATZ was measured in all samples collected. Atrazine is a corn herbicide that has been found to be ubiquitous in Iowa streams (Battaglin et al., 2005) because of the widespread application of ATZ that occurs annually across the entire state

(Fig. 1). With the exception of the two large border rivers sampled (i.e., Missouri and Mississippi Rivers), all basins in this study have between 50 and 80% of their land use in corn (areas that likely received recent ATZ applications) or soybean (areas that have likely received ATZ applications in previous years due to the common practice of corn–soybean planting rotation). For this study, ATZ was again found to be ubiquitous in Iowa streams, being detected in every sample collected (Table 3). Atrazine has been shown to exhibit an annual “spring flush” phenomenon in streams across the midwestern United States when stream concentrations substantially increase in the spring due to recent land applications of ATZ to fields planted with corn (Thurman et al., 1992). The results for this study followed this classic pattern for ATZ, with median concentrations substantially increasing in the early growing season (between March and June) and decreasing throughout the remainder of the growing season (Fig. 4a; Table 3). This temporal pattern of higher concentrations occurred during the early growing season (June) despite potential dilution from extreme flooding conditions that persisted across Iowa (Buchmiller and Eash 2010; Holmes et al., 2010). The ratio of DEA to ATZ (deethylatrazine to atrazine ratio [DAR]) provides a general indication of ATZ residence time (Adams and Thurman, 1991). The median DAR for this study decreased substantially between March (1.48) and June (0.28), corresponding with the fresh application of ATZ to fields recently planted to corn. The median DAR increased progressively through the rest of the growing season (July/August = 0.76, September = 0.89, October = 1.19) as ATZ continued to transform with time since application.

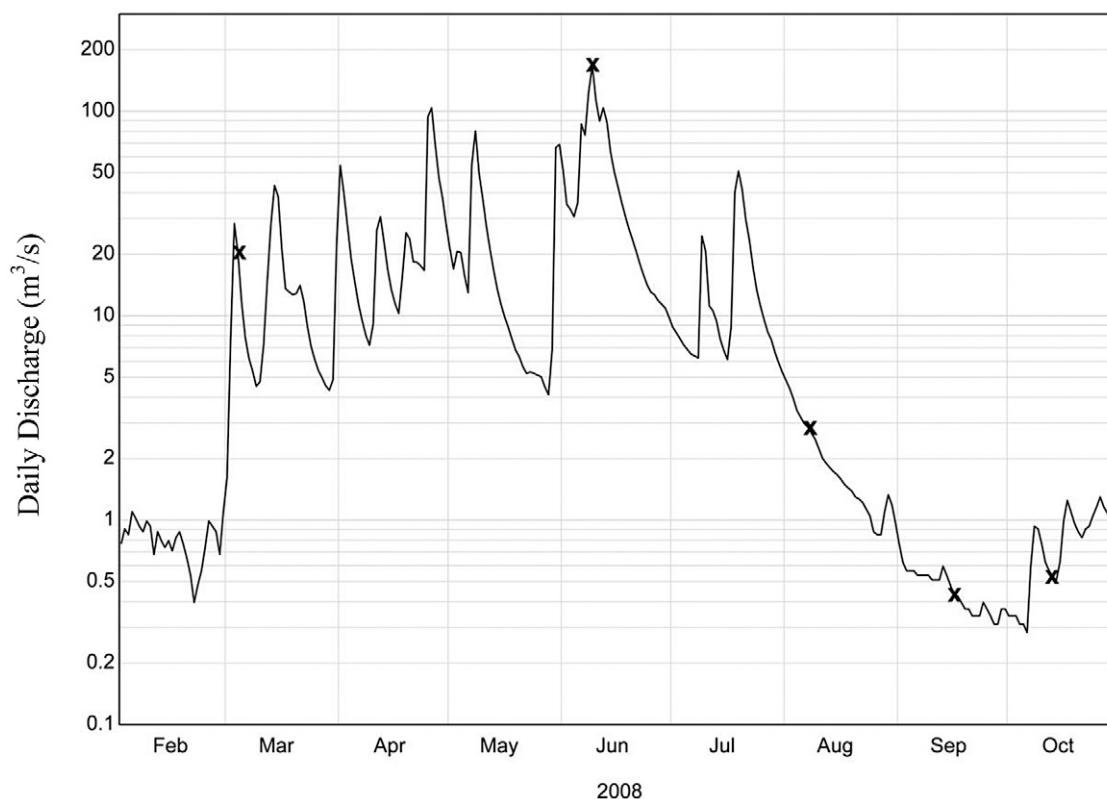


Fig. 3. Streamflow conditions at the South Fork Iowa River northeast of New Providence (05451210) during 2008 (USGS National Water Information System, 2009). The dates of sample collection for this site are denoted with an “X.”

Table 3. Summary results for the 75 stream samples collected in Iowa during 2008.† A total of 15 samples were collected during each sampling period, except where noted.

Compound	Mar.		June		July/Aug.		Sept.		Oct.		All samples	
	Max.	Freq.	Max.	Freq.	Max.	Freq.	Max.	Freq.	Max.	Freq.	Max.	Freq.
	ng L ⁻¹	%	ng L ⁻¹	%	ng L ⁻¹	%	ng L ⁻¹	%	ng L ⁻¹	%	ng L ⁻¹	%
Biochanin A	det	27	1.7	13	5.6	27	1.7	20	det	27	5.6	23
Coumestrol	ND	0	ND	0	ND	0	ND	0	ND	0	ND	0
Daidzein	41	73	det	7	10.5	27	23.6	7	22.69	47	41	32
Equol	40.4	93	3.2	20	9.5	40	12.9	20	5.07	53	40.4	45
Genistein	8	47	det	7	ND	0	ND	0	ND	0	8	11
Formononetin	12.3	93	5.3	93	13.5	87	12.1	40	13.15	87	13.5	80
Deoxynivalenol	583	53	–‡	–	ND	0§	26	60	11	47	583	43
Zearalenone	8	47	–	–	ND	0§	ND	0	ND	0	8	13
Atrazine	258	100	7300	100	347	100	240	100	305	100	7300	100
Deethylatrazine	212	100	1174	100	261	100	238	100	299	100	1174	100

† det, detected but not quantified; Freq., frequency of detection; Max., maximum concentration; ND, not detected.

‡ Not analyzed due to improper extraction.

§ Eleven samples were analyzed because four were improperly extracted.

Phytoestrogens

Similar to ATZ, phytoestrogens were commonly present in Iowa streams, with one or more of the five isoflavones being found in 85% of the 75 samples collected for this study. Formononetin was the most frequently detected target compound (80%), followed by equol (45%), DAI (32%), BIO (23%), and GEN (11%) (Table 3; Fig. 5). Coumestrol was not detected during this study. However, because an inverse relation occurs between the analytical reporting limit and a chemical's detection frequency (Kolpin et al., 1995), a truer comparison of detection frequencies can be made when a common detection threshold of 2.2 ng L⁻¹ is used (the highest reporting limit for the five isoflavones detected). After adjusting to this common detection threshold, the detection frequencies were: FOR (51%), equol (35%), DAI (19%), GEN (11%), and BIO (1%). This adjustment based on a common detection threshold only affected the ranking for BIO, where all but one detection were below 2.2 ng L⁻¹.

The five isoflavones detected had significant positive correlations (Spearman rank correlation; $p < 0.05$) with each other with the exception of GEN and BIO (0.006; $p = 0.96$) and GEN and FOR (0.15; $p = 0.20$), where no significant relation was determined. The isoflavone pairs most significantly related were FOR and BIO (0.56; $p < 0.0001$), FOR and DAI (0.53; $p < 0.0001$), and FOR and equol (0.51; $p < 0.0001$). Previous research of Swiss streams has also documented a positive relation between FOR and BIO (Hoerger et al., 2009). Contrary to this study, no other significant relations between isoflavone concentrations were observed in the Swiss data. This may be attributed to the substantial differences in land use between Iowa (dominated by corn and soybean) and Switzerland (dominated by clover). Significant negative correlations to ATZ were determined for DAI (–0.32; $p = 0.005$), and GEN (–0.28; $p = 0.02$). Atrazine is commonly used to control weeds in corn across Iowa but is not applied to soybean for such control. Thus, an inverse relation to isoflavones associated with soybean would be expected with a corn herbicide such as ATZ.

Because this study used an identical method for analyzing phytoestrogens as a previous study of Swiss streams (Hoerger

et al., 2009), select comparisons between the two studies can be made. Although the frequent FOR detections were similar to those found in studies of Swiss streams, BIO was detected at less than half the frequency (Hoerger et al., 2009). The second most frequently detected compound (equol) is an isoflavone metabolite that is not produced *in planta*. Equol can be formed internally in animals after plant consumption (Lundh, 1995; Heinonen et al., 2004). High concentrations of equol have been found in swine manure (Burnison et al., 2003), but equol was not detected in Swiss wastewater treatment plant effluent (Hoerger et al., 2009). The fact that Iowa is the largest producer of swine in the United States (USDA National Agricultural Statistics Service, 2009a) suggests that swine manure may be at least partially contributing to the equol concentrations documented in this study.

It was unexpected for FOR to be the most frequently detected compound in Iowa stream samples given the predominance of corn and soybean production (Fig. 1) compared with cover crops such as clover and alfalfa, which are known to contain high levels of FOR (Sivesind and Seguin, 2005; Seguin et al., 2004). The significant direct correlation of FOR to several isoflavones may be suggestive of multiple sources of FOR to streams, with the correlation to BIO indicating a potential clover source (Sivesind and Seguin, 2005; Seguin et al., 2004), the correlation to DAI indicating a potential soybean source (Maatooq and Rosazza, 2005), and the correlation to equol indicating a potential manure source (Burnison et al., 2003). More research is needed to specifically determine the primary sources and pathways of FOR that this study has shown to be frequently present in Midwestern streams.

Multiple isoflavones in a single water sample were common, with 57% of the samples having two or more, 31% having three or more, 15% having four or more, and 3% having all five isoflavones detected. Concentrations were low during the entire course of this study, however, with no concentrations exceeding 100 ng L⁻¹ for any of the 450 chemical isoflavone determinations for this study. This is in stark contrast to ATZ, where 42 of the 75 concentrations (56%) exceeded 100 ng L⁻¹, with 13 concentrations (17%) exceeding 1000 ng L⁻¹. Thus, the detected isoflavones were present at generally one to two

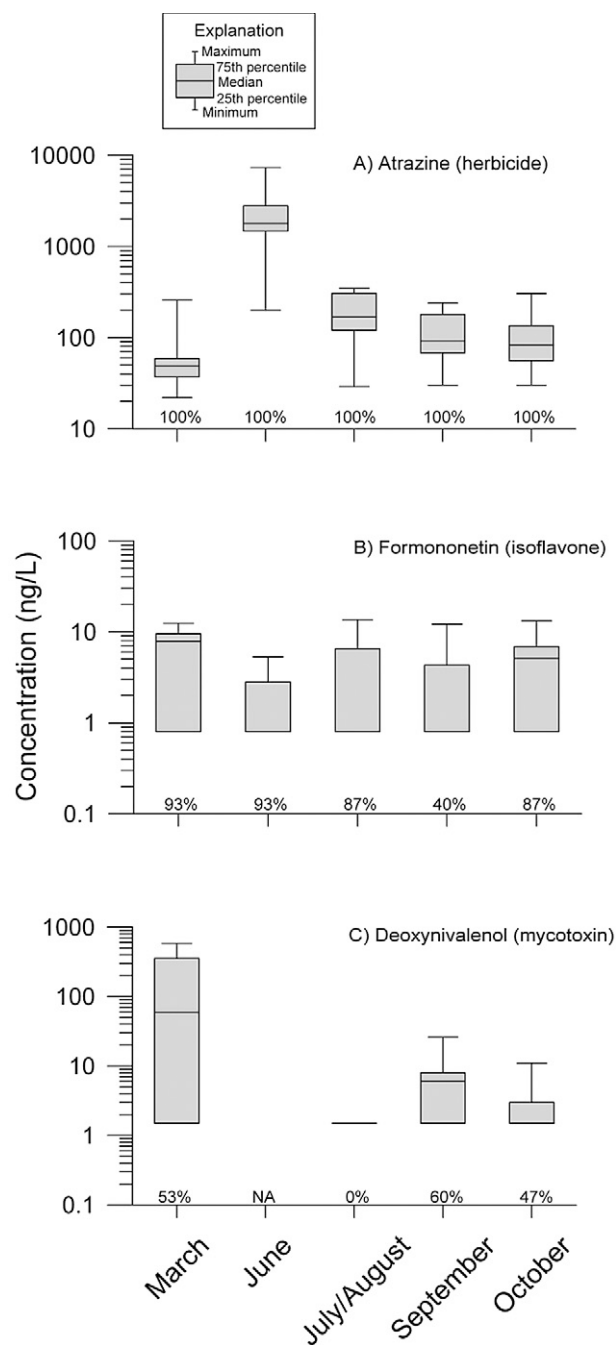


Fig. 4. Summary of chemical concentrations per collection period. (A) Atrazine (ATZ; limit of detection = 7 ng L⁻¹). (B) Formononetin (FOR; limit of detection = 0.8 ng L⁻¹). (C) Deoxynivalenol (DON; limit of detection = 1.5 ng L⁻¹). Percentages at the bottom of each panel indicate frequency of detection (NA = not analyzed because of improper extraction). Boxplots become truncated on the bottom end for compounds with low detection frequencies or look truncated for compounds with numerous detections at the reporting limit.

orders of magnitude less than ATZ (Fig. 5) even if all five compounds are summed into a total concentration.

The overall isoflavone results were generally similar among the sampling sites in terms of number of target compounds detected and total concentration even though basin area ranged four orders of magnitude (Table 1). The sites were divided into groups based on drainage area (<1000 km², 1000–10,000 km², 10,000–100,000 km², and >100,000 km²). No statistical difference was identified between these groups for the concen-

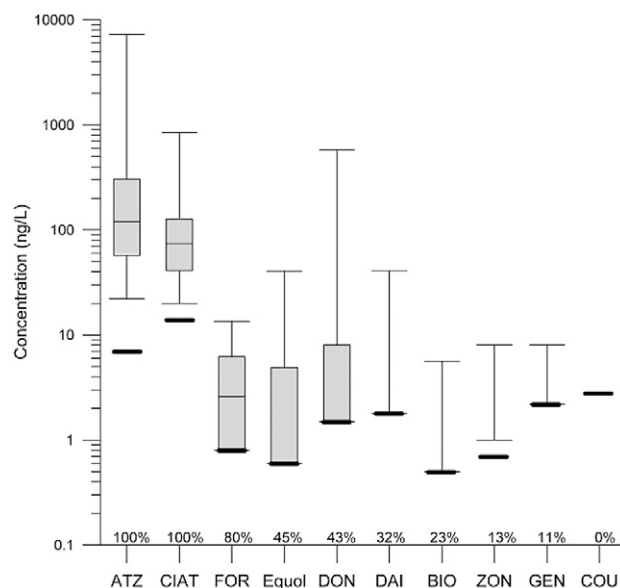


Fig. 5. Concentrations of target compounds detected in the 75 stream samples collected during 2008. Percentages at the bottom of each panel indicate frequency of detection. Bold line represents each compounds limit of detection. ATZ, atrazine (herbicide); BIO, biochanin A (isoflavone); COU, coumestrol (coumestan); DAI, daidzein (isoflavone); DEA, deethylatrazine (atrazine degradate); DON, deoxynivalenol (mycotoxin); equol (isoflavone); FOR, formononetin (isoflavone); GEN, genistein (isoflavone); ZON, zearalenone (mycotoxin). Explanation of a boxplot is provided in Fig. 4.

trations of individual isoflavones ($p > 0.05$; Kruskal–Wallis test), total number of target compounds detected ($p = 0.98$; Kruskal–Wallis test), or total isoflavone concentration ($p = 0.97$; Kruskal–Wallis test). The total isoflavone results ranged from a median of 0.8 ng L⁻¹ at the South Fork Iowa River headwaters (05451070) to 27 ng L⁻¹ at the Skunk River at Augusta (05474000).

Although the nearly ubiquitous detection of isoflavones during this study suggest a widespread source, such as the intense crop and livestock production that occurs across Iowa (Fig. 1), a significant relation was only determined between GEN and the percent of the basin in pasture/alfalfa (Spearman rank correlation = -0.25 ; $p = 0.04$). However, the extensive crop and livestock production across the entire state decreased the range in these potential explanatory factors (e.g., monoculture agriculture), limiting any significant relations between crop and livestock production to stream isoflavone concentrations.

To determine potential temporal patterns in stream isoflavone concentrations, FOR was examined because this was by far the most frequently detected isoflavone during this study. A temporal pattern was observed in stream FOR concentrations (Fig. 4b). The highest concentrations were observed in March during a period of snowmelt that increased streamflow across Iowa. These results suggest that isoflavones can accumulate in soils because plant residues degrade after a killing frost and crop harvest in the fall and because livestock manure is widely applied to the recently harvested fields. Such compounds can then be mobilized and transported to streams during winter snowmelt conditions. Substantially lower isoflavone concentrations were observed in the June collection. This was likely due to a combination of the flushing of chemical residues from the system during the previous period of snowmelt conditions

and subsequent spring rainfall runoff, the early plant stage occurring at this time of the growing season, and the extreme flooding conditions (Buchmiller and Eash, 2010; Holmes et al., 2010) that persisted across Iowa at this time. The median streamflow of sampled sites increased by over an order of magnitude during June from the previous sampling period in March (Table 1). Dilution during higher streamflows has been documented previously for isoflavones (Hoerger et al., 2009). Isoflavone stream concentrations generally increased from the July/August to the October collections (Fig. 4b) as crops matured to the harvest stage (Fig. 2). In addition, manure applications were likely taking place in some areas during October as harvest progressed. However, lower concentrations were observed during September when the lowest streamflows of the study were encountered (Table 1; Fig. 3). The dry conditions during this period likely limited the transport of isoflavones to streams through pathways such as overland flow and tile drains. The results of this study suggest that runoff, crop growth, and manure application conditions are important factors in determining stream concentrations for isoflavones. Thus, a runoff event early in the growing season (June flood) can express the same pattern of lower stream isoflavone concentrations as a prolonged dry period later in the growing season (September baseflow).

Although ATZ and FOR are primarily agriculturally derived, substantial differences in their input functions translate to stark contrasts in the temporal patterns in stream concentrations between these contaminants (Fig. 4). The generally single, pre-emergent application of ATZ to corn leads to the repeatedly documented pattern of greatly increased stream ATZ concentrations after application associated with corn planting and progressive decreases through the remainder of the growing season. However, FOR is primarily derived from a combination of plant production and the application of livestock manure to fields. Thus, a more complicated temporal pattern to stream concentrations is to be expected. However, the ubiquitous detection frequencies of ATZ and FOR suggest that both have a widespread source in such agriculturally dominated stream systems.

Mycotoxins

The mycotoxins DON and ZON were detected in 43% of the 56 samples analyzed. However, DON was detected over three times more frequently than ZON (Table 3; Fig. 5) despite the higher reporting levels for DON (1.5 ng L^{-1}) compared with ZON (0.7 ng L^{-1}). Although few studies have investigated the occurrence of DON and ZON in streams, previous research has documented a similar increased prevalence of DON compared with ZON (Bucheli et al., 2008). Maximum DON concentrations for this study were substantially greater than maximum ZON concentrations and about an order of magnitude higher than previously reported in Swiss river waters (Bucheli et al., 2008). The two mycotoxins were positively correlated (0.63 ; $p < 0.0001$, Spearman rank correlation), as one would expect from compounds derived from a similar source and formation process. Zearalenone was always detected in the presence of DON. Concentrations were generally low during the entire course of this study, with only 6 out of 112 (5%) chemical mycotoxin determinations exceeding 100 ng L^{-1}

(109, 229, 355, 465, 507, and 583 ng L^{-1}), all of which were for DON. Although sharply less than the number of concentrations exceeding 100 ng L^{-1} for ATZ (42/75), DON was the only natural contaminant for this study that had individual concentrations that exceeded the median concentrations measured for ATZ (Table 3; Fig. 5).

The overall mycotoxin concentrations were generally similar among the sampling sites even though basin area ranged four orders of magnitude (Table 1). No statistical difference was identified among the four previously defined groups based on drainage area size for DON concentrations ($p = 0.07$; Kruskal–Wallis test), ZON concentrations ($p = 0.50$; Kruskal–Wallis test), total number of mycotoxin compounds detected ($p = 0.08$; Kruskal–Wallis test), or total mycotoxin concentration ($p = 0.07$; Kruskal–Wallis test). There were apparent differences among individual sampling sites, however, with two sites having no mycotoxins detected (05418600, 05420680), seven sites where mycotoxins were detected in 25 to 50% of the samples, four sites with 51 to 75%, and two sites where mycotoxins were detected in every sample collected (06609500, 06810000). Three sites had median DON concentrations exceeding 10 ng L^{-1} (06609500 = 12 ng L^{-1} , 06607500 = 13 ng L^{-1} , and 05474000 = 18.5 ng L^{-1}). Unlike ATZ and FOR, DON detections were not ubiquitous across Iowa (Table 3). In fact, a spatial pattern was observed where basins draining the western half of Iowa (05474000, 05490500, 06485500, 06607500, 06609500, and 06810000; Fig. 2) were those where DON was frequently detected (e.g., in >50% of the mycotoxin samples collected). The only site draining areas of the western half of Iowa where DON was not frequently detected was site 06610000 (1 of 4 samples, Missouri River at Omaha; Fig. 1) where a substantial portion of this basin contains land areas outside of Iowa. The importance of this finding is not that the western part of Iowa had higher DON concentrations but that DON did not display the same ubiquitous pattern across the state, as was shown with FOR (Fig. 4). The less spatially widespread detections of DON compared with ATZ and FOR suggest a more variable source because not only is a host required (e.g., corn or wheat) but also the proper soil temperature and moisture conditions to promote *Fusarium* spp. infections. The use of fungicides in crop production may also be important in determining stream mycotoxin concentrations. Fungicide use in Iowa has increased in recent years due to the concern of soybean rust that was first documented in the United States in 2004 (Livingston et al., 2004) and the trend in crop management practices to use fungicides as a mechanism to increase crop yields (Bradley and Sweets, 2008).

The largest instantaneous DON load observed for this study was $320 \text{ million ng s}^{-1}$ (0.32 g s^{-1} ; 06610000, Missouri River at Omaha). Extrapolating these instantaneous levels over 24 h would translate to the transport of almost $28,000 \text{ g}$ of DON in a single day. This is three orders of magnitude greater than loads determined from wastewater treatment plants in Switzerland over an entire week (Wettstein and Bucheli, 2010). Thus, the amounts of mycotoxins being transported by streams draining agricultural areas can be substantial.

Similar to that for phytoestrogens, the extensive crop and livestock production across the entire state (Fig. 1) decreased the range in these potential explanatory factors (e.g., monoculture

agriculture), limiting significant relations between crop and livestock production to stream mycotoxin concentrations. The only significant relation observed was between DON and the percent of the basin in soybean (0.32; $p = 0.03$; Spearman rank correlation). Soybean is not a host plant for *Fusarium* spp. infections. Thus, this may be a spurious relation because there is no apparent explanation for the relation between DON and soybean production.

Mycotoxin concentrations exhibited a temporal pattern much different from ATZ or FOR (Fig. 4). The highest mycotoxin concentrations were observed in March (Table 3). This is the first time that DON and ZON have been documented to persist in soil or crop residues through the winter and to be transported to streams during snowmelt conditions. In addition, this is the only sampling period where ZON was detected and when DON concentrations exceeded 100 ng L⁻¹ (Table 3). The spring flush of mycotoxins is likely a result of the combination of weathering and partial degradation of mycotoxin-containing crop residues and ongoing mycotoxin production of fungi on these residues until temperatures drop with the onset of winter conditions. Unfortunately, improper extraction affected all of the samples from the June collection and four of the samples from July/August collection. However, neither DON nor ZON were detected in the 11 remaining samples available from the July/August collection. This suggests that the combination of the flushing of chemical residues from the system during the snowmelt conditions occurring in March followed by the extreme flooding conditions occurring in June (Fig. 2 and 3) decreased mycotoxin concentrations to below the detection limit during the July/August collection. During the September collection, DON was the only target compound that had an increase in detection frequency (0% in July/August to 60% in September) (Fig. 4). This increased occurrence of DON may be related to the combination of warm air and soil temperatures, crop maturity, and wet soils that occurred earlier in the summer, which were the proper conditions for increased *Fusarium* spp. infections in corn and wheat. Although the DON detection frequency in September was similar to that in March (Fig. 4), the DON concentrations were generally 10 times higher in March. The corresponding higher flows in March compared with September (Table 1) translated to DON stream loads being about 26 times higher in March compared with September. Deoxynivalenol concentrations and frequencies of detection decreased from September to October (Table 3). This is in contrast to the trends observed for ATZ (similar concentrations) and FOR (increasing concentrations) during these two sampling rounds (Fig. 4). More research is needed to determine the causes for this late growing season decrease in DON.

Conclusions

Phytoestrogens and mycotoxins are naturally occurring compounds that are derived from a wide variety of plant and fungal species, many of which are known to have estrogenic properties. Little research has been done to determine their environmental distribution. This study found that, although phytoestrogens and mycotoxins were commonly detected in rivers and streams across Iowa, concentrations were typically <50 ng L⁻¹. Because the estrogenic potencies of these compounds are generally sev-

eral orders of magnitude less than 17 β -estradiol (Coldham et al., 1997; Matthews et al., 2000), considerably larger concentrations would be expected to reach a comparable estrogenic effect. Although the data from this study suggest that such concentrations are not likely to occur frequently in Iowa streams, the DON results indicate that concentrations may approach these levels under certain hydrological conditions. During the March sampling conducted during snowmelt conditions, 40% of the DON concentrations exceeded 100 ng L⁻¹, with 13% exceeding 500 ng L⁻¹. This is the first time that elevated concentrations of DON has been documented in streams during such snowmelt conditions, although more research is needed to determine if this is an annual phenomenon. The ecotoxicological impacts from the long-term, low-level exposures to natural contaminants such as phytoestrogens and mycotoxins or complex chemical mixtures, including these compounds and the plethora of other agriculturally derived contaminants (e.g., pesticides, hormones, veterinary pharmaceuticals, etc.), that are known to also be commonly present in streams, however, are poorly understood and have yet to be systematically assessed in environmental studies. Thus, a better understanding of the spatial and temporal occurrence of natural contaminants is important for assessing potential cumulative impacts of such compounds and broader chemical mixtures on aquatic biota as well as their potential presence in drinking-water sources.

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