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Endocrine Disrupting Compounds and Smallmouth bass (*Micropterus dolomieu*) in the Juniata River: a frontier in fisheries research

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Abstract

The Susquehanna River basin is one of the largest and most diverse watersheds in the northeastern United States, draining nearly half of Pennsylvania. *Micropterus dolomieu* (smallmouth bass- SMB) are the predominant sportfish throughout the Susquehanna and Juniata River Basins, and recent work suggests that SMB populations in these watersheds have been on the decline for over a decade. Runoff of agricultural herbicides has been identified as one of the major health risks for SMB populations in the Susquehanna River Basin, especially due to their potential effects as endocrine disrupting compounds (EDCs). During the summers of 2016 and 2017, we assessed potential impacts of agriculture on SMB populations in 21 tributaries to the Juniata River. Passive water samplers were installed for 39-40 days at six sites with varying levels of agricultural intensity. Samplers were screened for six common agrochemicals (atrazine, metolachlor, acetochlor, prometon, simazine, desethylatrazine) with atrazine being the most common. Additionally, SMB (n=66) were collected from nine sites in 2016 and 2017 to assess their health and morphology. Blood samples were collected from SMB in 2017 and assessed for vitellogenin presence in blood plasma where presence was confirmed in 100% of male (n=11) and female (n=11) SMB from five sites. A digital morphology assessment of whole body shape indicated significant differences between several sites that we relate to two different feeding strategies: suction and ram feeding. Since EDCs affect fish taxa greater than macroinvertebrates, piscivorous SMB with ram feeding morphologies are more vulnerable to potential food web alteration from EDCs. Overall, our watershed wide study of the Juniata River Basin suggests strong agricultural influence in light of EDCs, with the potential for degradation of the smallmouth bass fishery at the ecosystem level, and should be considered in the scope of the Susquehanna and Chesapeake Bay watersheds.

Introduction

The Susquehanna River basin is one of the largest and most diverse watersheds in the northeastern United States, draining nearly half of Pennsylvania, and supports both coldwater and warmwater fisheries (Mcllnay 2002). The Susquehanna River fishery is renowned for its smallmouth bass (*Micropterus dolomieu*) fishing and was ranked 76th in the United States in 2012 (Bassmaster 2012). Within the larger Chesapeake Bay, smallmouth bass fishing has an annual economic impact of 630 million dollars (Allen et al. 2013). The Juniata River is the second largest tributary to the Susquehanna River, which contributes 43% of the Chesapeake Bay watershed (Langland et al. 1995). Draining 3,400 square miles, the Juniata River basin is comprised of forested ridges, agriculture in the valleys, and a few urban centers (Lefevre 2005). Historical land use practices in the Juniata River basin included logging, coal mining, agriculture, as well as steel production, all of which have had their respective environmental impacts (Mcllany 2002). Currently the entire Susquehanna watershed has been under intense focus due to its degradation from a previously premier smallmouth bass fishery. Recent surveys by the Pennsylvania Fish and Boat Commission (PAFBC) have indicated smallmouth bass population decline, especially in the young of year (YOY), juvenile age class, in almost all sections of the Susquehanna and Juniata Rivers since the early 2000's (Smith 2010; Arway & Smith 2013). Therefore, in December 2015 in coordination with PAFBC, the Pennsylvania Department of Environmental Protection (PADEP) published the Causal Analysis/ Diagnosis Decision Information System (CADDIS) report, which detailed potential perturbations to the smallmouth bass population (Shull & Pulket 2015). The CADDIS report highlighted 14 candidate causes for population declines throughout the Susquehanna Basin, with EDCs and herbicides, as well as pathogens and parasites as the two most likely stressors on smallmouth bass health. The report further discussed major research gaps essential to understanding the basin's smallmouth bass decline in population and overall health.

Endocrine disrupting compounds (EDCs) from a wide array of sources threaten the sustainability of freshwater ecosystems across the world from organismal to population levels (Tyler et al. 1998). EDCs can pollute streams from both point and non-point sources such as wastewater treatment effluent, and agricultural runoff, respectively (Kirk et al. 2002; Vajda et al. 2008, Folmar et al. 2001). EDCs are especially of concern because municipal wastewater treatment plants are incapable of removing these compounds from their effluent, and therefore EDCs are rarely removed from the environment (Aerni et al. 2004; Sarmah et al. 2006). Further, EDCs possess the ability to mimic estrogen, thereby reducing the reproductive viability of aquatic male organisms across several taxa (Bernanke & Köhler 2008; Kortenkamp 2007). Effects from EDCs such as intersexing, reduced gonadal development and viability, and skewed sex ratios have all been shown to have detrimental effects on fish at the individual and population level (Kaptaner et al. 2009; Blazer et al. 2014). Further, relationships between intersexing and proximity to waste water treatment plants, as well as industrial sources like paper mills, have been made across the United States (Liney, et al. 2005; Woodling 2006, Sepúlveda et al. 2003), and even within the Susquehanna River basin (Blazer et al. 2014). However, non-point sources, such as agricultural runoff, have been much less studied and is suggested to be a more complex interaction (Mann et al. 2009). Therefore, agricultural EDCs in the Juniata River is a research gap especially relevant to the smallmouth bass population and health decline, and further needs to be studied.

EDCs from herbicides and pesticides often enter waterways through runoff from agriculture operations. While much effort has been made to implement best management practices (BMPs) to reduce the transport of agricultural chemicals to waterways, there is still a high prevalence of these chemicals in nearby waterways, both freshwater and saltwater (Thurman et al. 1992; Steen et al. 1999). Temporally, the transport of these chemicals is well studied, as it's been established that their entrance to the aquatic ecosystem is most often the result of episodic rainfall events, especially in the spring (Stoeckel et al. 2012; O'Brien et al. 2016). The overlap between spring-time agrochemical applications and increased precipitation results in pulses of agricultural chemicals entering nearby streams through overland flow runoff. While temporally the transport of agrochemicals to streams is well established, much debate continues on the most influential environmental and physical factors (land use, slope, in-stream vegetation, application rate, etc.) effecting agrochemical transport at the watershed scale (Huber et al. 2000; Lagacherie et al. 2006; King et al. 2008; Smiley et al. 2012; Dabrowski et al. 2002). Since agrochemical transport is complex, the most effective predictive models often utilize a multivariate approach with predictive factors often varying among watersheds and season (Lagacherie et al. 2006).

EDCs in the form of herbicides are a major concern for the United States due to their prevalence yet mixed consensus of environmental impacts (Rohr & McCoy 2010; Renner 2004; Hayes 2004; Giddings et al. 2005; Huber 1993; Solomon et al. 1996, 2008). Atrazine, which is outlawed in the European Union, is arguably the most significant EDC in the United States at this time, as it is the most widely used herbicide on broadleaf crops such as corn, soybeans, and sorghum (Bethsass & Colangelo 2006; Kiely et al. 2004). Presence of atrazine in watersheds across the United States has been confirmed to be as high as 92% with the 90th percentile being above 5 ug/L, all taking place in early June (Solomon et al. 1996). Culminating the aforementioned studies, the EPA has concluded concentrations of atrazine at 10-20ug/L is likely to have both population and community level effects, and therefore they set a chronic aquatic community benchmark of 17.5 ug/L, regardless of other countrys' significantly lower benchmarks such as Canada's 1.8 ug/L (Canadian Council of Ministers of the Environment 1999; USEPA 2011).

EDCs ability to mimic estrogen thereby having the potential to induce vitellogenisis in male fishes and other taxa is the mode that causes cascading reproductive and population effects. In male fishes, concentrations of vitellogenin, a protein precursor of egg yolk, are naturally very low (Iwanowicz & Blazer 2011). However, when fish are exposed to EDCs, regardless of the source, they have been widely shown to express increased vitellogenin concentrations, even to levels comparable of female fishes (Kirby etal. 2004; Harries etal. 1996; Harries etal. 1997; Cheek etal. 2001; Hinck et al. 2007). Other studies have further verified abnormal vitellogenin concentrations using a 17ß-estradiol and testosterone ratio (Toft et al. 2003). Therefore, detection of vitellogenin can be effective in assessing potential EDC exposure among aquatic male organisms.

With a lack of studies investigating agricultural herbicides' potential influence from the watershed to organismal level, we sought out to fill this research gap in relevance to the Juniata River basin. Our goal was to focus on agriculture's effect using multiple advanced ecological indicators within the Juniata River, in hopes of furthering our understanding of the smallmouth bass decline in both population and health. Using the Juniata River basin as a model for the rest of the Susquehanna watershed, our study design was focused on detailing how EDC presence in major tributaries may have effects on fish health in the form of intersexing and morphological changes. Our results suggests that high EDC concentrations from agricultural herbicides threaten the sustainability of smallmouth bass populations within the Juniata Basin, by contributing to high prevalence of intersexing, and potentially stressing morphologically vulnerable smallmouth bass population by affecting trophic dynamics.

Materials and Methods

Site Selection

The Juniata River basin is situated in the larger Susquehanna River and Chesapeake Bay watersheds. Sites (n=21) within the Juniata Basin were selected to assess varying land covers present across the basin (figure 1). Sites and their respective watersheds were initially selected, using aerial imagery from Google Earth, to estimate land cover categories for sites of interest. Sites were also targeted to broadly assess the largest tributaries to the Juniata River, several locations on the main-stem Juniata River, and evenly distribute across the basin. Each tributary was later delineated using 30m DEM projections and various hydrology tools in ArcGIS 10.5 (Environmental Systems Research Institute 2013). Land cover statistics (% row crop, % forested, etc.) were calculated for each watershed using the 2011 National Land Cover Database (Homer et al. 2015). Other watershed characteristics (basin area, slope, and stream density) were calculated using the USGS StreamStats data tool (USGS 2012).

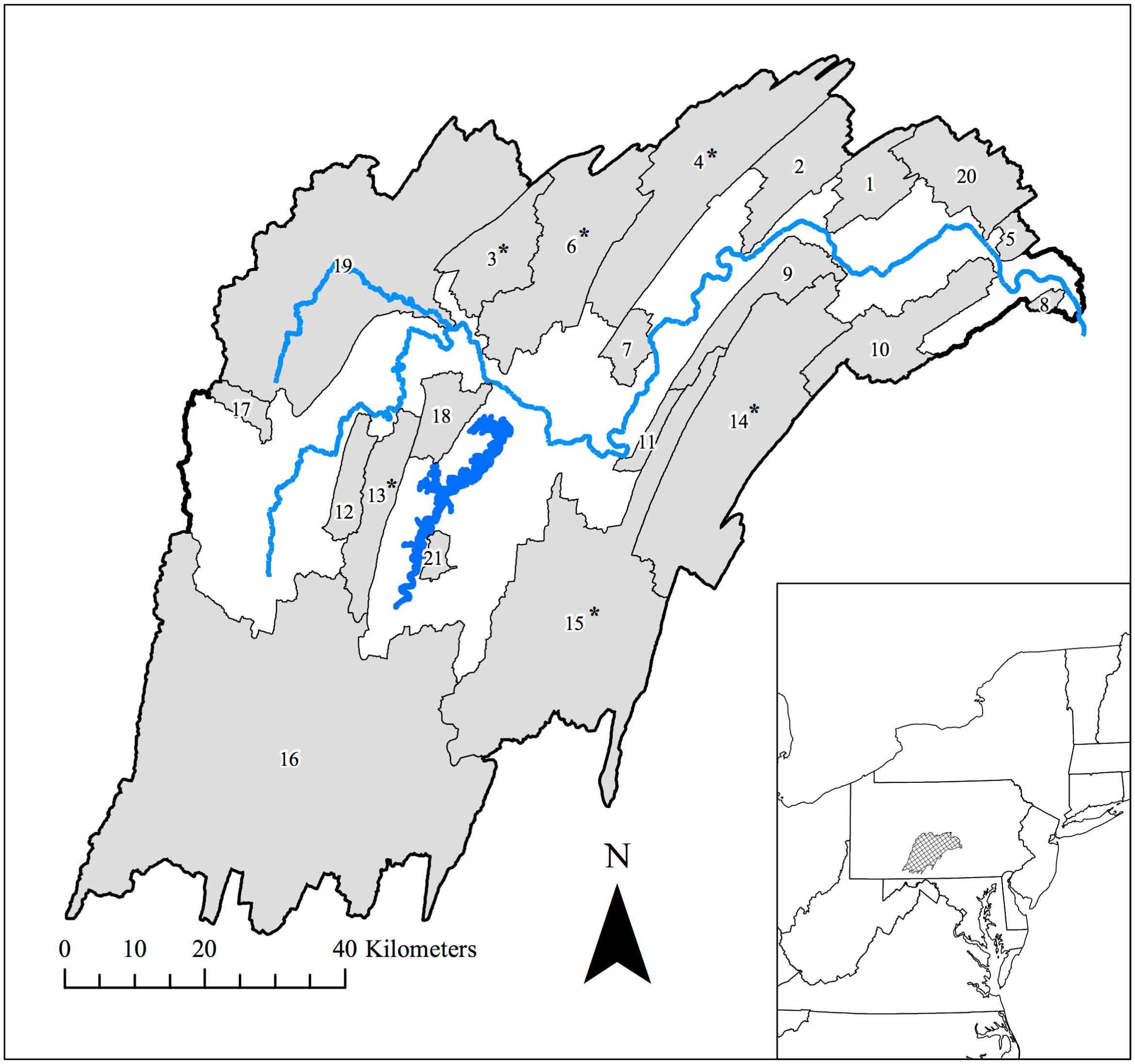

Figure 1. Map of the Juniata River basin, Pennsylvania with sampled watersheds numbered and shaded grey. Passive water sampler sites are asterisked. Raystown Lake and main-stem Juniata River are shown in blue.

Water chemistry

Physiochemical parameters including stream pH, dissolved oxygen, temperature, conductivity, total dissolved solids (TDS), and salinity were measured at all 21 tributaries in the summer of 2016 using a Hanna Multiparameter probe (HI9813-6). Additionally, water samples in 2016 were collected at all sites and analyzed for phosphorus using Seal AQ2 (EPA-134), and chlorophyll-a (SM10200) (APHA 2005). A subsample of 11 streams was assessed in 2017 for nitrate/nitrite

(SM4500-NO3 E-00), and phosphorus (SM4500-NO3 E-00) in order to further investigate sites that indicated potentially high agricultural influence the previous year (APHA 2005). Finally, historic (2010) nitrate, nitrogen, and phosphorus data was compiled from USGS and EPA sampling that overlapped our tributary sampling (US EPA 2018).

Passive Water Samplers

Passive water samplers, Polar Organic Chemical Integrative Samplers (POCIS), were deployed (n=6) by anchoring the samplers to the streambed with 1 meter long rebar and metal wire for 38-39 days between May 31st and July 8th 2016 (figure 2). A field blank was used during installation and recollection to address potential contamination as samplers were handled and prepared outside the stream environment. Passive water samplers were chosen for sampling because they capture the pulse like dynamics herbicides exhibit when entering streams. After the deployment period, passive water samplers were collected and immediately stored on ice. Samplers were then shipped to Environmental Sampling Technologies (St. Joseph, Montana, USA) for sample extraction. Samples were extracted with 25 mL Methanol, blown down using ultra high purity nitrogen gas, and then the extracts from each of the three sampling membranes were pooled. Pooled extracts were then blown down again, filtered through glass fiber filter paper with Methanol, and transferred to yield 5mL of concentrated pooled extract from each site. Extracts were then sent for analysis by Anatek labs (Moscow, Idaho) for a screen of 5 herbicides and one metabolite (atrazine, metolachlor, alachlor, simazine, prometon, and desethylatrazine (DEA)) according to EPA 8270CMOD methods (EPA 2018). We used the quantified chemical masses to estimate in-stream herbicide concentrations according to Mazzella et al. 2007:

$$C_w = \frac{N}{R_s t}$$

Where…

C_w is the amount of the ambient chemical concentration of sampled site (ng/L)
N is the amount of the chemical accumulated by the sampler (ng)
R_s is the chemical specific sampling rate (L/d)
t is the exposure time (d)

Figure 2. Picture of passive waters sampler, Polar Organic Chemical Integrative Samplers (POCIS), with three sampling membranes used to estimate in-stream herbicide concentrations at six sites across the Juniata Basin in the summer of 2016

Fish Collection

Smallmouth bass (n=66) were collected from 8 sites during the summers of 2016 (n=37) and 2017 (n=29) via a combination of backpack electrofishing and traditional angling techniques. Captured fish were sacrificed via immersion in Tricaine MS-222, following humane procedures and in compliance with Juniata College IACUC #2016-04-002 (National Research Council 2010). Post-euthanasia, fish were pinned to a foam board to splay each fin, and photographed with a standard length in the frame for later digital morphology analysis (figure 3) (Seiler et al. 2009). Blood was sampled from smallmouth bass collected in 2017 using heparinized capillary tubes and cardiac puncture. Collected blood was chilled on ice for further in lab analysis. Fish were also sexed in the field, and testes (if present) were removed and stored in 10% neutral buffered formalin.

Figure 3. Smallmouth bass sample from main-stem Juniata River, Pennsylvania pinned to foam board for later digital morphology analysis (2017)

Vitellogenin and testicular oocyte analysis

Within 12 hours of collection, blood samples were centrifuged at 3000 rpm for 5 minutes or until there was a clear diversion between red blood cells and plasma. Centrifuged capillary tubes were measured for hematocrit (red-blood volume / total blood volume). Capillary tubes were then scored using a file and broken to isolate the blood plasma and transferred to microtubes. A 1:1 dilution was made for each sample using sample buffer and then samples were placed on a heat block at 100º C for 4 minutes. After heating, samples were loaded and ran through a 7.5% 15-well SDS-PAGE electrophoresis gel alongside a pair of molecular weight markers. Gels were stained with Brilliant Blue for 18-24 hours on a rocker table then viewed on a light box for presence of vitellogenin around 200kDa (Orlando et al. 1999). Testes stored in formalin were further processed to assess potential testicular oocytes using established methods (Tetreault et al. 2011). In short, processing the testes included imbedding teste tissue in paraffin, cross-sectioning tissue to 5µm using a microtome, mounting to a microscope slide, and staining using Hematoxolin and Eosin (H&E).

Data Analysis

For digital morphological analysis, 15 homologous landmarks were placed on each image (figure 3), and scales were set using the StereoMorph package in R-studio (R Core Team 2015). With landmarks in place, digital frameworks of each sample were exported from StereoMorph and uploaded to MorphoJ for statistical analysis (Klingenberg 2011). A full Procrustes superimposition was used to remove the effects of size, rotation, and translation on point placement (Dryden & Mardia 1998). A covariate matrix was generated on Procrustes coordinates, then analyzed using a canonical variate analysis (CVA) with stream site as a classifier group, and a permutation test of 100,000 pairwise distances was used on the CVA to yield p-values for site comparison. Sites within one stream mile of each other were combined after initial analysis showed strong overlap between geographically close sites, and further literature supported one stream mile was within smallmouth bass's home range (Munther 1970; Gatz & Adams 2015). Wire frame graphs were generated for each canonical variate to visualize morphological differences. Procrustes coordinates, averaged by site, were exported from MorphoJ to further analyze morphological data with site-based data (water chemistry, land cover, etc.). Statistics for all other variables were verified for normality using Shapiro-Wilk test, and analyzed for correlations in R-studio with significance considered at $\alpha < 0.05$.

Results

Physiochemical Parameters

Physiochemical data was highly varied across the Juniata Basin, with a lack of significant correlations between a single land cover or watershed characteristic (agriculture, development, forest, basin slope) (table 1). Total dissolved solids ranged between 54.9 and 455ppm (n=21), salinity between 39.5 and 307ppm (n=21), nitrogen between below detection and 37.1 mg/L (n=11), and phosphorus between below detection and 0.186 mg/L (n=11). Physiochemical data (TDS, nitrate, phosphorus, etc.) was not significantly correlated to percent agriculture or any other land use characteristics within the sampled watershed (P> 0.05). There was however, a general trend across multiple physiochemical parameters often associated with agricultural influence (TDS, Nitrogen, Phosphorus, etc.) (figure 4).

Table 1. Site statistics of Juniata River tributaries, Pennsylvania (n=21) sampled in June and July of 2016 and 2017. Sites where passive water samplers (POCIS) were deployed are shown with asterisks (n=6). Land uses are expressed in percentages, basin slope in degrees, stream density in miles stream/sq. miles, and basin area in km^2.

Site #	Site	Lat.	Long.	Row crop	Pasture/hay	Forest	Developed	Basin slope	Stream density	Basin area
15	Aughwick*	40.27801	77.88708	3.64	10.47	79.59	5.79	10.4	1.91	788.443
10	Buffalo	40.49429	77.13874	7.89	19.01	65.69	6.43	8.5	2.06	184.249
13	Clover*	40.47237	78.1726	28.74	13.78	51.55	5.85	7.2	1.83	126.817
20	Cocolamus	40.53711	77.1464	16.41	14.17	62.88	5.75	8.2	1.86	165.03
18	Crooked	40.4831	78.01599	6.47	16.85	67.99	8.55	10.3	2.17	80.2114
9	E. Licking	40.52859	77.3932	4.83	9.31	81.85	3.83	11.4	1.91	118.608
2	Jacks	40.5847	77.5564	10.01	13.55	70.17	5.85	9.2	1.53	155.205
14	Kishacoquillas-upper*	40.66113	77.59624	18.16	12.36	62.07	7.2	9.5	1.43	419.556
19	Little Juniata	40.56119	78.068161	12.22	8.7	69.32	9.13	8.2	1.59	881.735
1	Lost	40.58996	77.40602	13.5	18.53	60.18	6.94	7.9	1.53	102.435
17	Mill	40.46649	78.418736	0.41	2.34	57.15	39.01	7.8	1.67	33.7976
7	Musser	40.49282	77.74155	14.67	14.23	64.21	6.48	9.2	1.38	47.9928
12	Piney	40.47242	78.23005	32.03	12.22	50.41	5.25	6.5	1.86	65.7014
16	Raystown Branch- upper	40.19191	78.25471	9.15	16.95	65.9	7.37	9.1	2.33	1917.87
3	Shavers*	40.58244	78.04584	8.38	12.58	74	4.56	9.6	1.92	146.468
6	Standing Stone*	40.4919	77.99398	4.47	5.82	84.49	4.86	9.9	1.72	341.117
21	Tatman	40.30502	78.16959	1.11	5.26	88.76	4.6	10.8	2.1	20.107
14	Tuscarora*	40.52818	77.39329	7.47	12.33	75.47	4.12	10.6	2.02	550.058
11	W. Licking	40.35947	77.81738	0	1.06	96.22	2.69	15.4	0.93	28.9809
8	White	40.45556	77.03204	2.08	11.99	77.44	8.49	12.1	2.53	9.97926
5	Wildcat	40.51426	77.12966	20.79	15.85	56.14	6.86	8.2	1.67	24.206

Table 2. Site statistics for main-stem Juniata River, Pennsylvania sites (n=7) sampled in summers 2016 and 2017. Land uses are expressed in percentages, basin slope in degrees, stream density in miles stream/sq. miles, and basin area in km^2.

Site	Lat.	Long.	Row crop	Pasture/hay	Forest	Developed	Basin slope	Stream density	Basin area
Juniata-Duncannon	40.402767	77.013656	9.12	12.5	69.54	7.59	9.4	1.94	8792.76
Juniata-McVeytonwn	40.49738	77.73899	8.29	11.88	70.73	7.82	9.4	2	6364.73
Juniata-Mifflintown	40.59441	77.41491	8.91	11.93	70.04	7.88	9.4	1.94	7227.04
Juniata-Millerstonw	40.53632	77.14764	9.17	12.27	69.77	7.58	9.5	1.94	8428.37
Juniata-Newton Hamilton	40.39198	77.83441	8.14	11.84	70.9	7.86	9.4	2.01	6215.53
Juniata-Petersburg	40.53987	78.03367	11.61	10.59	66.91	10.21	8.9	1.79	2080.5
Juniata-Huntingdon	40.47819	78.00289	10.33	10.03	69.41	9.58	9	1.79	2538.19

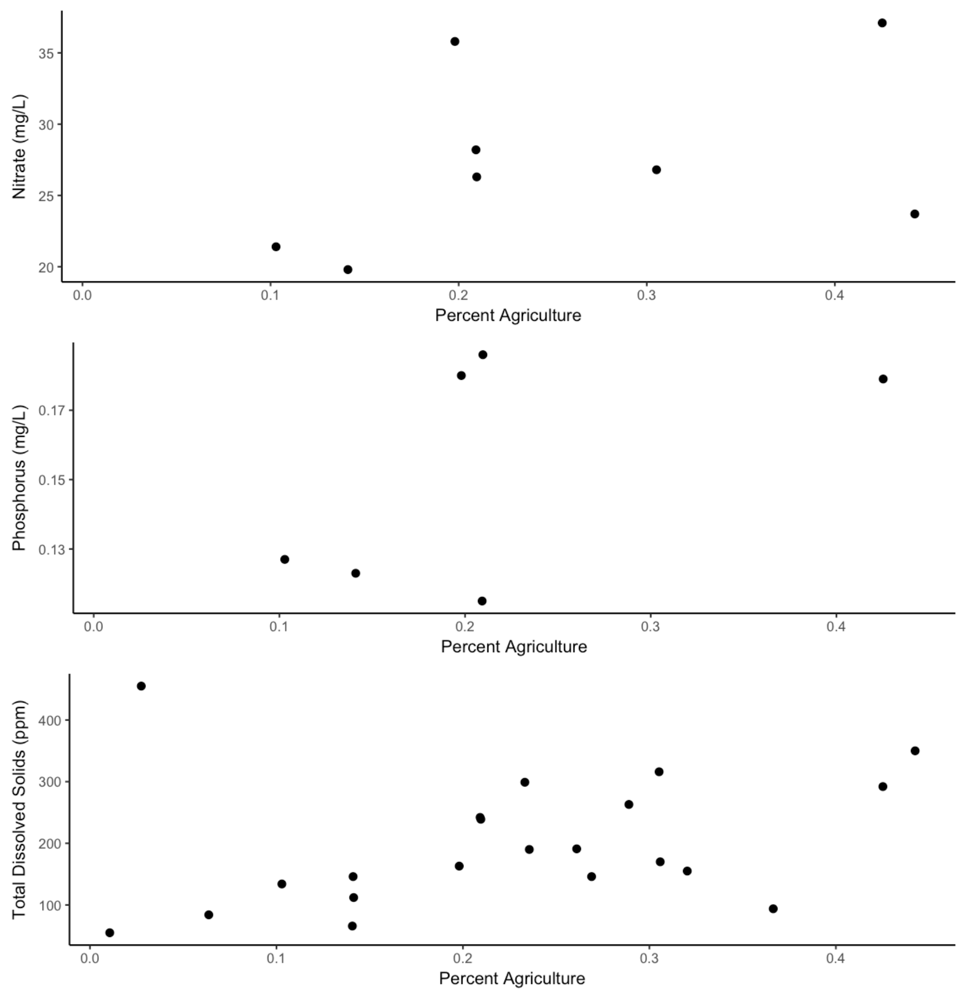

Figure 4. Scatter plots of A) Phosphorus versus % Agriculture (P=0.2599, r= 0.548) B) Nitrate versus % Agriculture (P= 0.3164, r= 0.407) C) TDS versus % Agriculture (P= 0.2046, r= 0.289) of Juniata River tributaries, Pennsylvania in June and July 2016 and 2017

Herbicides

Across the Juniata River basin, herbicide presence was confirmed at all six of the assessed sites, with varying concentrations between sites (figure 5). Atrazine was the only chemical confirmed at all sites ranging between 11.09 and 91.02 ng/L, with Tuscarora having the highest concentration. Additionally, metolachlor was confirmed at five of the six sites ranging between 3.47 to 47.45 ng/L, again with Tuscarora having the highest concentration (table 3). Only one site had all six herbicides, at relatively low levels, and that was Kishacoquillas. Although atrazine was quantified at all sites, two sites were found to be absent of its respective metabolite desethylatrazine (Tuscarora and Standing Stone). Statistically, atrazine was not significantly correlated with a single land use or other watershed characteristic (percent agriculture, acreage of row cropland, watershed slope, watershed density), but showed a general positive trend betweeb stream density, and the total area of cropland in the watershed (figure 6).

Table 3. Estimated agrochemical concentrations (ng/L) from POCIS sampling in Juniata River tributaries, Pennsylvania for 38-39 days between May 31st and July 8th 2016. Dash represents samples below detection limit.

Site	Atrazine	DEA	Simazine	Metolachlor	Acetochlor	Prometon
Shavers	38.26	6.92	-	11.13	-	-
Kishacoquillas	32.7	23.51	5.56	11.7	2.27	5.25
Standing	11.09	-	-	-	-	-
Clover	67.17	33.43	-	3.47	-	-
Tuscarora	91.02	-	54.72	47.45	-	-
Aughwick	55.42	3.78	-	25.92	-	-

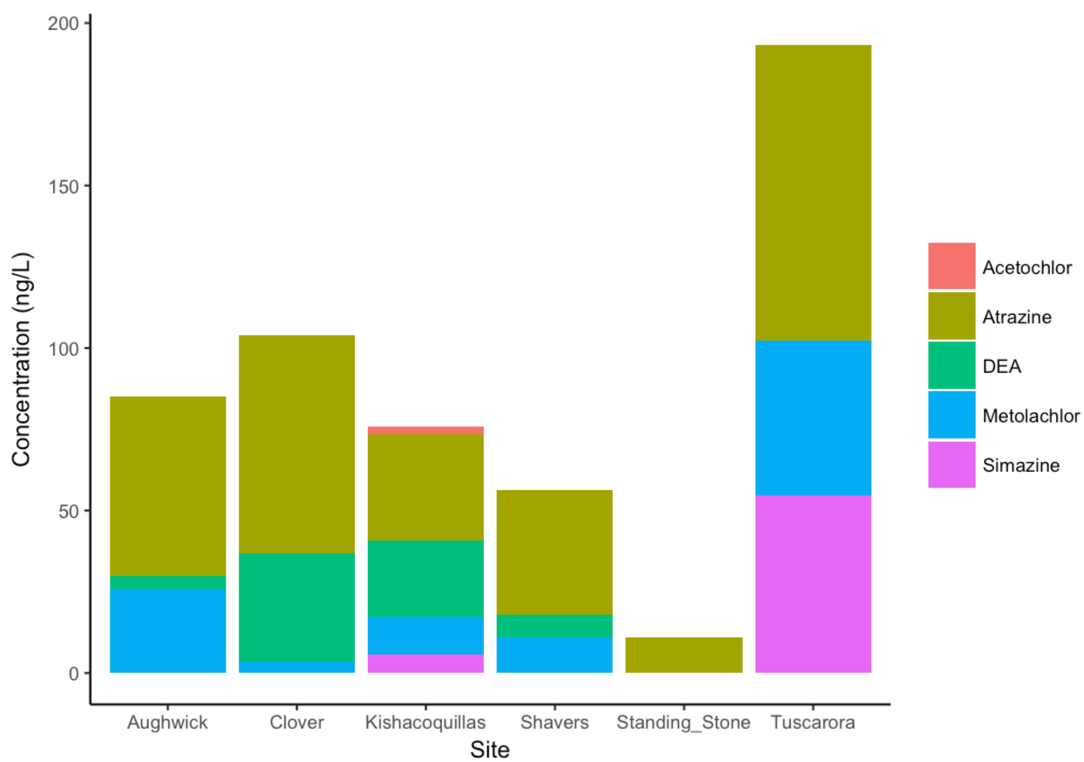

Figure 5. Agrochemical concentrations for tributaries sampled with passive water samplers (POCIS) in the Juniata River basin, Pennsylvania for 38-39 days between May 31st and July 8th 2016

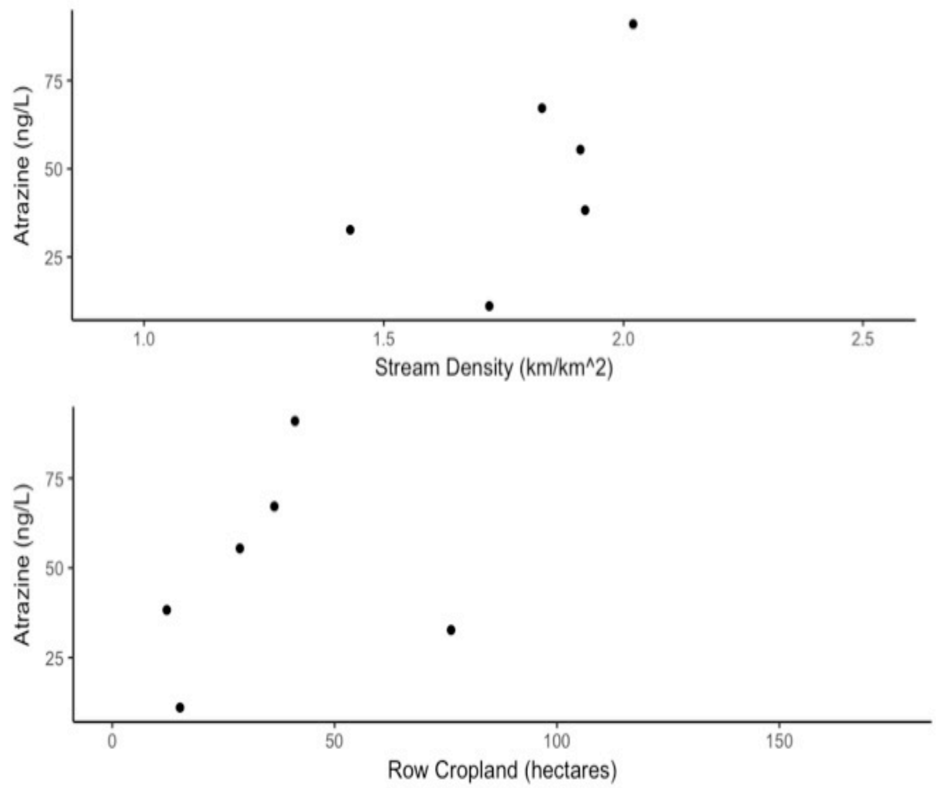

Figure 6. Estimated atrazine concentrations from passive water samplers versus A) stream density ($P = 0.1886$, $r = 0.621$) and B) area of row cropland ($P = 0.7432$, $r = 0.173$). Samplers were installed for 38-39 days between May 31st and July 8th 2016 in Juniata River tributaries, Pennsylvania.

Digital Morphology

Morphological analysis was conducted between smallmouth bass sub-populations (n=9) within the Juniata River basin with sample sizes ranging from 4 to 19 smallmouth bass per site. The analysis indicated statistically significant variation in morphologies between several sites (figure 7). Overall, 62.96% of the variation amongst the dataset was accounted for by the first two CVs in the CVA. Many sample sites overlapped in morphologies, while several other sites including Aughwick, Petersburg, and Huntingdon proved to be the most different (figure 7 & table 4). Largely, there appears to be a divergence in smallmouth bass morphology separated at the zeros of CV1 and CV2. As indicated by the wire frame graph of CV1 where most of the variation is explained (43.14%), the major morphological differences are present in the jaw and caudal peduncle region, with the positive end of CV1 having smaller jaws and wider caudal peduncles. Although site averaged procrustes shapes were analyzed for correlations with all of the physiochemical, herbicide, and watershed characteristic data we collected, none of them proved to be significant in influencing the morphologies. The possibility of sex influencing body morphology was assessed utilizing a CVA of overall body shape with sex as a factor, as well as specific sex linked characteristics including upper and lower jaw length, but each proved to be insignificant; P=0.6910, P= 0.9311, and P=0.7692 respectively.

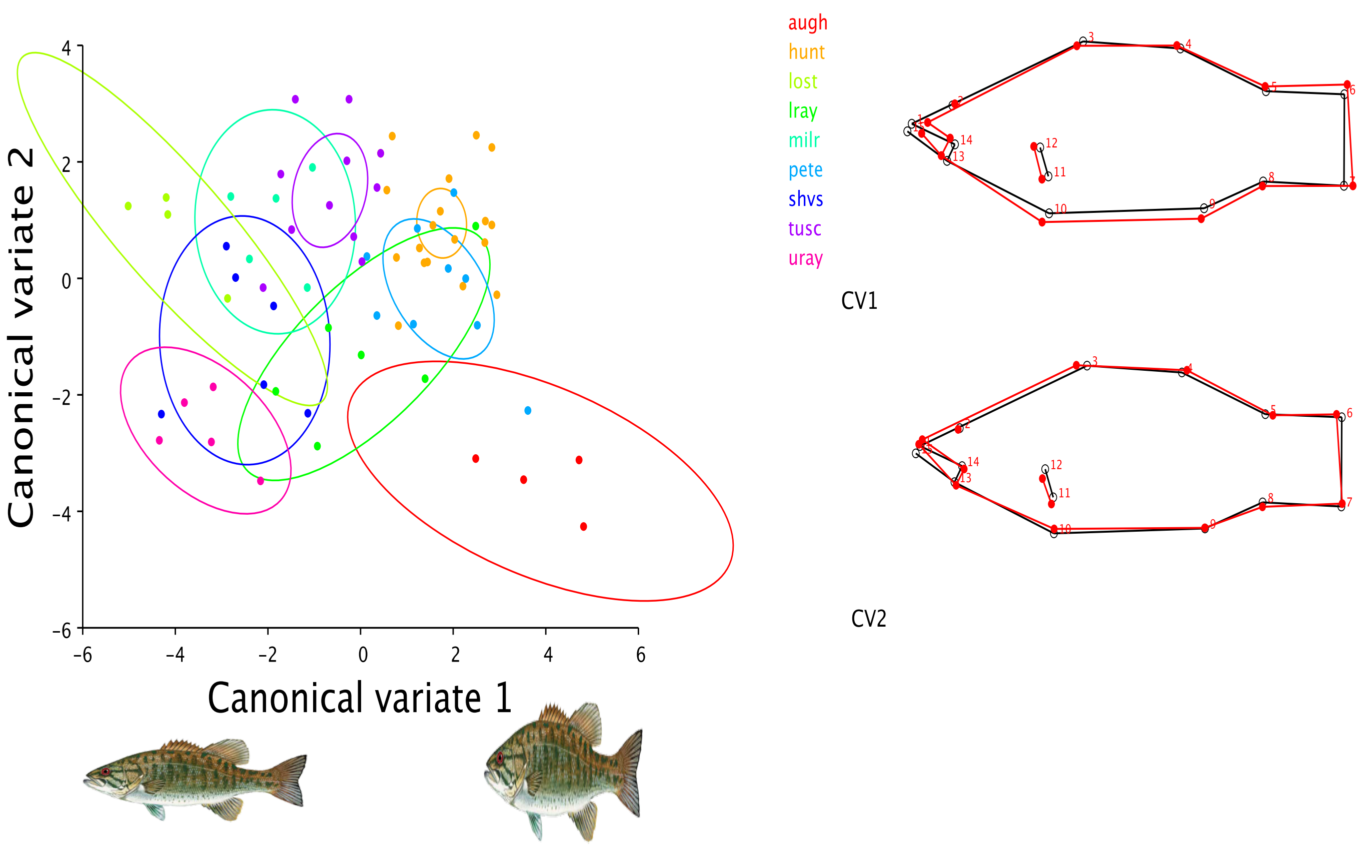

Figure 7. Morphological canonical variate plot (CV1 & CV2) with respective wireframe graphs for smallmouth bass (n=69) sampled from the Juniata River basin, Pennsylvania in the summers of 2016 & 2017. CV1 and CV2 account for 62.96% of the entire sample's variation. Ellipses are drawn to 95% confidence intervals. Pictograph shows the major morphological changes that occur on the extremes of CV1.

Table 4. P-values from permutation tests (100,000 permutation rounds) for Procrustes distances among groups on canonical variate analysis. (CVA) Asterisks denote P-values significance, where * < 0.05, ** < 0.01, *** < 0.001.

	Aughwick	Huntingdon	Lost	Lower Rays.	Millerstown	Petersburg	Shavers	Tuscarora
Huntingdon	0.0417*							
Lost	0.0148*	0.0034**						
Lower Rays.	0.4562	0.334	0.1177					
Millerstown	0.0225*	0.0007***	0.307	0.0815				
Petersburg	0.1212	0.2482	0.0033**	0.2159	0.005**			
Shavers	0.0156*	0.0212*	0.3863	0.8428	0.1466	0.0478*		
Tuscarora	0.0296*	0.1166	0.1251	0.2516	0.0338*	0.0116*	0.2041	
Upper Rays.	0.0416*	0.0013**	0.378	0.3093	0.1959	0.0027**	0.551	0.107

Histology

Presence of vitellogenin in blood plasma was confirmed in 100% of male (n=11) and female (n=11) smallmouth bass collected in 2017. Unfortunately teste tissue samples collected for oocyte analysis were compromised and rendered unusable. Hepatosomatic index (HSI) was calculated on a subsample of smallmouth bass (n=50) from fish collected in both 2016 and 2017. Site based characteristics were not shown to be significant factors in HSI; rather it proved to be sex based. Male smallmouth bass had statistically lower HSI (n= 26, average = 0.0101) than female smallmouth bass (n= 24, average = 0.0161) (P < 0.001) (figure 8).

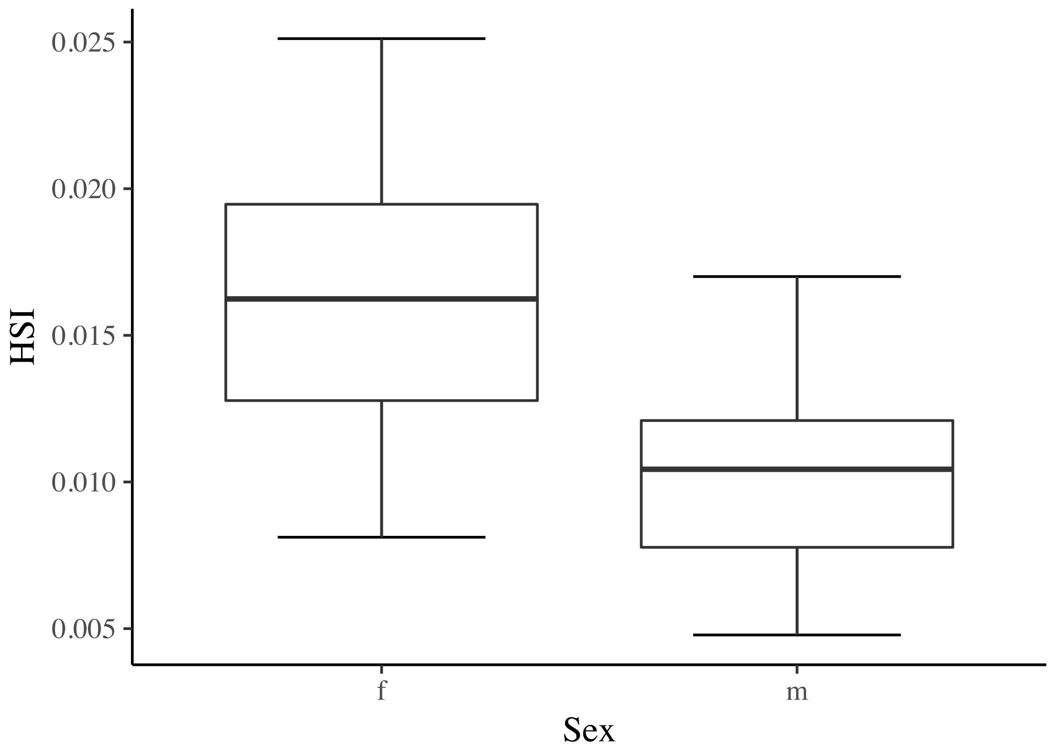

Figure 8. Boxplot of smallmouth bass hepatosomatic index (HSI) in females (n=24) versus males (n=26) sampled from the Juniata River basin, Pennsylvania in the summers of 2016 & 2017 (P < 0.001).

Discussion

Agrochemical concentrations in this study of the Juniata River basin were found to be of biological concern. Most notably, atrazine and metolachlor were confirmed at detectable levels at six and five sites respectively (table 2). The agrochemicals assessed in this study, especially atrazine and metolachlor, have substantial evidence as EDCs and their respective quantified concentrations have the potential to negatively effect fish and other taxa at the population and individual level (Hayes et al. 2010; Hayes et al. 2011; Tillitt et al. 2010; Kolpin et al. 2012; Hillis et al. 2015). Further, atrazine concentrations in our study of Juniata River tributaries were found to be upwards of 3 times higher than those found in previous sampling of the mainstem Juniata River, but similar to that of other tributaries to mainstem rivers across the Chesapeake Bay basin (Blazer et al. 2014, Kolpin et al. 2013; Walsh et al. 2018). Compared to previous studies, the higher concentrations in this study are likely related to the sizes of watersheds we sampled as it has been noted that concentrations of agrochemicals are highest in mid-sized tributaries, and lowest at the extremes of watershed size (Richards & Baker 1993). One study in particular, Hall et al. 1999, highlighted this dynamic in the Chesapeake Bay basin where both atrazine and metolachlor concentrations were at their respective minimum in the main-stem bay and river, but at their maximum at the mid-sized stream scale (Hall et al. 1999). It is therefore suggested that this highlights the dilution that occurs with increased watershed size. In light of the agrochemical concentrations we documented as well as others in the Chesapeake Basin, we further support there is a peak in agrochemical concentrations in tributaries, and a dilution in main-stem rivers.

The increased agrochemical concentrations we documented in tributaries have potential implications for reproductive and developmental stress on smallmouth bass populations. Tributaries are essential spawning and refuge habitat for smallmouth bass in late spring and early summer (Humston et al. 2010, Gerber & Haynes 1988, Edwards et al. 1983). It is further suggested that the spawning period is the most influential time in a year class's success for smallmouth bass population recruitment (Clancey 1980). Exposure to agrochemical concentrations at the juvenile life stage poses a severe risk, as it is smallmouth bass's most vulnerable time period (Walsh et a. 2018). Therefore, the high concentrations of atrazine and metolachlor we found in the essential spawning grounds of Juniata River tributaries can have serious reproductive complications such as intersexing for smallmouth bass populations (Kolpin et al. 2013).

High prevalence (100%) of intersexing in our study further supports previous work in the Chesapeake Bay watershed, as well as for the black bass family. Previous studies in watersheds across the Chesapeake Bay basin including the mainstem Juniata, Potomac, Swatara, Shenandoah, and Susquehanna Rivers all have documented evidence of intersexing (vitellogenin presence and testicular oocytes) as high as 100%, and included both smallmouth bass and white suckers (Blazer 2014, Alvarez et al. 2008; Lee Pow et al. 2017; Blazer et al. 2007; Blazer et al.. 2012). Further, several reviews have found centrachids to be one of the most intersexed families, with smallmouth bass being the most prevalently intersexed species (Abdel-Moneim et al. 2015; Iwanowicz et al. 2016; Hinck et al. 2009). The sensitivity of this family has been suggested to be due to differences in molecular and cellular process that allow sexual differentiation to be influenced by EDCs, regardless of the source (wastewater, agriculture, urban) (Sepúlveda et al. 2003; Woodling et al. 2006; Vajda et al. 2008; Tetreault et al. 2011). Chemicals including household/personal care products, prescriptions, industrial chemicals, herbicides, and insecticides all have been shown to effect sexual expression within the black bass family (Kolpin et al. 2013). It is suggested that these chemicals have severe reproductive implications including reduction in sperm and egg quality and quantity, and an overall significant retardation of reproductive systems (Fuzzen et al. 2015; Abdel-Moneim et al. 2015; Hecker et al. 2006). Additionally, histological measures including testicular oocytes have been used to further look at organelle level effects of EDCs. Egg development within the tissue of testes in male fish has also been related to measures of reproductive vitality (Blazer et al. 2007). Therefore, in the Juniata Basin, high agrochemical concentrations of EDCs like atrazine in essential spawning habitat of smallmouth bass highlights a potential explanation for poor recruitment and population numbers. While our study only highlights six EDCs, it is highly likely there are more EDCs at concentration of concern in many tributaries. Therefore, not only should baseline intersexing be of concern, but also so should EDC effects at the reproductive level. As the CADDIS report outlines, EDC exposure is one of the two most probably causes for smallmouth bass population decline in the Susquehanna River Basin, and their role in decreasing or halting reproductive potential is a likely mode of degradation, especially in light of their increased concentrations around spawning habitat.

Hepatosomatic index (HSI), an indicator of pollutant exposure, differentiated between sexes, highlighting potential effects of EDCs within the Juniata Basin. Female smallmouth bass were shown to have statistically significant higher HSI than male smallmouth bass (figure 8). However, this relationship seems to be atypical of smallmouth bass, especially given studies often do not partition between sexes in statistics around HSI measures, rather focus on site-based characteristics (% agriculture, EDC concentrations) or biological measures (oocyte index, gonadosomatic index, vitellogenin levels) (Pinkney et al. 2017). Previous work has shown a positive relationship between HSI and EDCs or other contaminant exposure in smallmouth bass, as well as other fishes, as EDCs directly affect the synthesis of vitellogenin (Hinck et al. 2009; Fishelson 2006; Prado et al. 2011; Donohoe et al. 1999). However, in our dataset, land use characteristics did not prove to be a significant driver of HSI as sex was highly significantly different, and thereby caused too much site based variation. Further, HSI from sampled female smallmouth bass appeared to be elevated compared to other studies (Lee Pow et al. 2017; Pinkney et al. 2017). While sex based differences in HSI is generally rare, a few studies did similarly find an increase in exposed female HSI while males remain constant (Lee Pow et al. 2017; Sepúlveda et al. 2003; O’connor et al. 2013). More specifically, one study noted smolt-age atlantic salmon experienced sex based differences in HSI, females exhibiting higher HSI, post exposure to atrazine for 21 days at concentrations comparable to those in the Juniata Basin (100 ug/L) (Nieves-Puigdoller et al. 2007). VTG production was confirmed in male samples in this study, and elevated HSI further supports potential vitellogenisis in our sampls. Therefore our study provides a unique insight into this relationship in that it could suggest potential hyper vitellogenin activity in female smallmouth bass within the Juniata Basin. Further studies should investigate potential application of HSI as an indicator of sexual degradation, especially in important game fish.

Additionally, the agrochemical concentrations in this study highlight how multiple factors contribute to the variability in agrochemical concentrations within Juniata River tributaries. While our study is limited by its sample size, figure 6 shows a general positive trend between atrazine and two watershed characteristics 1) basin stream density and 2) the acreage of row cropland in a watershed. Agrochemicals, especially atrazine, degrade much faster in terrestrial environments than aquatic, and therefore a higher stream density watershed will have proportionally higher agrochemical concentrations (Solomon et al. 1996). Additionally, the amount or proportion (percent) of row cropland has been well established as the primary influence of agrochemical concentrations in streams. The amount of agrochemicals in a stream is limited by the total amount of agrochemicals applied within the watershed, and therefore is a primary influence on in-stream concentrations, as shown in its significance as a factor in many multiple regression models (Hall et al. 1999; Lagacherie et al. 2006; Lerch et al. 2011; Smiley et a. 2012). In relation to our study, the complexity in agrochemical interplay generally followed suit with previous studies in the Chesapeake basin and beyond, but would require more sample sites to fully support.

Morphological differences in smallmouth bass populations in tributaries to the Juniata River suggest potential vulnerabilities in light of EDCs. As shown in figure 7, morphologies across sampled tributaries was highly varied, with several significant differences occurring between sites. Specifically, our results suggest a divergence in morphologies with sites on the positive side of CV1 (Aughwick, Huntingdon, and Petersburg) exhibiting larger jaws and thinner caudal peduncles. In contrast, the other sites were found to have smaller jaws, wider body depths, and thicker peduncles, of which both morphologies have potential feeding strategy implications. Two types of feeding strategies are common amongst predatory fishes including ram and suction feeding. Ram feeding is frequently associated with chasing smaller fishes and requires thinner caudal peduncles and larger jaw gapes in order to accelerate to top speed and capture quickly fleeing prey. In contrast, suction feeding morphology includes wider caudal peduncles with smaller jaw gapes in order to better maneuver and inhale smaller prey items in and around in-stream cover like macroinvertebrates (Bloodworth & Marshall 2005; Norton & Brainerd 1993; Norton 1995; Wainwright & Richard 1995; Wainwright et al. 2001; Faye et al. 2012). Differences among morphologies between tributaries highlights different feeding strategies, and should be investigated alongside EDC exposure.

Previous studies have shown that EDCs affect vertebrate taxa more readily than invertebrate taxa, thereby making vertebrate populations more at risk of population decline (Farruggia et al. 2016). Food webs in the Juniata River include adult smallmouth bass as a top predator, feeding on smaller fish as well as crayfish and other macroinvertebrates (Buynak et al. 1982; Johnson & Dropkin 1993, 1995). Given EDC pressures on baitfish prey items, smallmouth bass utilizing this resource are potentially vulnerable, especially given the previously mentioned differences in morphologies and feeding strategies. Potential loss or decrease in baitfish prey items are likely to force adult smallmouth bass to rapidly adjust their diets to macroinvertebrates. To do this effectively, smallmouth bass feeding strategies would need to change to suction feeding, a genetic and phenotypic trait that takes several generations to adapt, depending on population size (Lande 1986). Therefore, sites with morphologies best suited for ram feeding including Aughwick, Huntingdon, and Petersburg, are all at risk due to EDCs potential affect on baitfish populations. Cascading effects from EDCs altering food web dynamics and predator prey relations could prove to be a potential risk for smallmouth bass population sustainability. In this sense, further research should address more widespread EDC concentrations in essential spawning habitats, effects of EDCs beyond prevalence of intersexing by investigating direct effects to sexual expression and reproductive success, and potential ecosystem vulnerabilities to EDC presence in the form of altering food web dynamics.

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