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Relation of contaminants to fish intersex in riverine sport fishes

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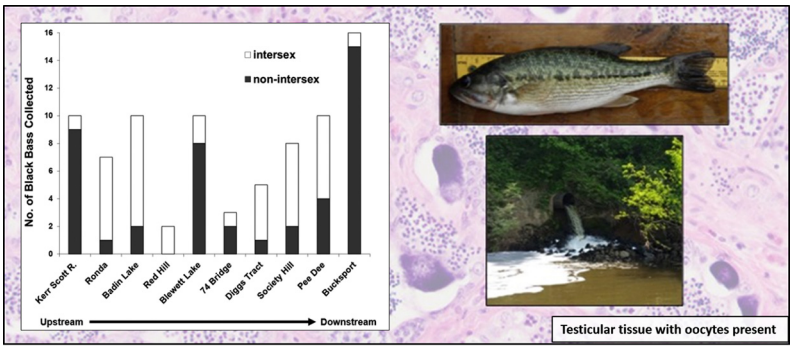
HIGHLIGHTS

- Fish intersex is a common condition related to endocrine disrupting contaminants.
- Contaminant concentrations and fish intersex are rarely examined simultaneously.
- Fish, water, and sediment were evaluated in a large southeastern USA regulated river.
- Intersex occurrence was greatest in black bass and varied throughout the river.
- PAHs, mercury, and pesticides correlated most with fish intersex.

GRAPHICAL ABSTRACT



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ABSTRACT

Endocrine active compounds (EACs) are pollutants that have been recognized as an emerging and widespread threat to aquatic ecosystems globally. Intersex, the presence of female germ cells within a predominantly male gonad, is considered a biomarker of endocrine disruption caused by EACs. We measured a suite of EACs and assessed their associated impacts on fish intersex occurrence and severity in a large, regulated river system in North Carolina and South Carolina, USA. Our specific objective was to determine the relationship of contaminants in water, sediment, and fish tissue with the occurrence and severity of the intersex condition in wild, adult black bass (*Micropterus*), sunfish (*Lepomis*), and catfish (Ictaluridae) species at 11 sites located on the Yadkin-Pee Dee River. Polycyclic aromatic hydrocarbons (PAHs), ethinylestradiol (EE2), and heavy metals were the most prevalent contaminants that exceeded effect levels for the protection of aquatic organisms. Fish intersex condition was most frequently observed and most severe in black basses and was less frequently detected and less severe in sunfishes and catfishes. The occurrence of the intersex condition in fish showed site-related effects, rather than increasing longitudinal trends from upstream to downstream. Mean black bass and catfish tissue contaminant concentrations were higher than that of sunfish, likely because of the latter's lower trophic position in the food web. Principal component analysis identified waterborne PAHs as the most correlated environmental contaminant with intersex occurrence and severity in black bass and sunfish. As indicated by the intersex condition, EACs have adverse but often variable effects on the health of wild sport fishes in this river, likely due to fluctuations in EAC inputs and the dynamic nature of the riverine system. These findings enhance the understanding of

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the relationship between contaminants and fish health and provide information to guide ecologically comprehensive conservation and management decisions.

1. Introduction

Aquatic ecosystems are under threat because of their susceptibility to act as sinks or conduits, accumulating and transporting numerous chemical contaminants released from industrial, agricultural, and municipal sources (Scholz and Mayer, 2008). Such sources of contaminants may become increasingly detrimental to aquatic species, due to persistent human population growth and pollution. One class of contaminants that has been identified as a significant stressor is endocrine active compounds (EACs). The endocrine system is important to the health and reproductive success of organisms, and alterations to it can be harmful to individuals and populations (Lee Pow et al., 2017b; Solomon, 2015). EACs may interfere with the endocrine system by disrupting normal synthesis, storage, release, metabolism, transport, binding action, and elimination of endogenous hormones; those implicated in such disruption are recognized as endocrine disrupting compounds (EDCs; Kavlock et al., 1996). Further, with exposures at sufficient levels, or during critical times of development, EACs have the potential to cause toxicity to an organism (Barton and Andersen, 1998).

EACs that are often introduced into aquatic systems include polychlorinated biphenyls (PCBs), current use pesticides (CUPs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), pharmaceuticals, bisphenol A (BPA), and heavy metals, forming chemical mixtures with potential additive or synergistic effects (Hinck et al., 2009; Muthumbi et al., 2003). EACs vary widely in their persistence in aquatic environments, and those resistant to environmental degradation are classified as persistent organic pollutants (POPs); common POPs in aquatic systems are PCBs and OCPs, which are known for persistent legacy effects on biota (El-Shahawi et al., 2010; Harding et al., 1998). Originating from numerous routes of entry and anthropogenic influences, EACs have been detected in aquatic environments globally and are likely to remain ever-present with associated ecological effects (Abdel-Moneim et al., 2015; Kolpin et al., 2002; Ternes et al., 1999).

The estrogenic potency of some EACs has been cause for concern because of the potential for negative effects on reproduction and sustainability of wildlife populations. EACs have been linked to skewed sex ratios, reduced fecundity, production of vitellogenin (an egg-yolk precursor) in male fish, intersex condition, and population collapse in fish (Bhandari et al., 2015; Brian et al., 2007; Jobling et al., 1998; Kidd et al., 2007; Lee Pow et al., 2017b; Puy-Azurmendi et al., 2013; Williams et al., 2009; Woodling et al., 2006). Intersex, the presence of female germ cells within a predominantly male gonad (Nolan et al., 2001), has been suggested as a biomarker of endocrine disruption from environmental, and especially estrogenic EACs (Bahamonde et al., 2013; Barnhoorn et al., 2004; Leino et al., 2005). To assess the impacts of EACs on wildlife, intersex has been evaluated in various wild fish populations worldwide (Adeogun et al., 2016; Allen et al., 1999; Bizarro et al., 2014; Blazer et al., 2007; Tetreault et al., 2011). By examining intersex and EACs, researchers are able to better understand the dynamics and health of an ecosystem. Fish are particularly applicable for this area of study because they are susceptible to EACs through the aquatic environment, can act as sentinel species, and are indicators of ecosystem health (Kolpin et al., 2002; Kwak and Freeman, 2010; Lee Pow et al., 2017b; Penland et al., 2018).

In the southeastern United States (USA), fish intersex has been documented in numerous fish populations (Hinck et al., 2009; Kellock et al., 2014; Lee Pow et al., 2017a; Penland et al., 2018). This region is especially relevant for the study of EAC effects on fish because of impacts caused by dense human populations, agriculture, industry, wastewater treatment, and concentrated animal feeding operations, which have all been described as significant sources of EACs (Kolpin et al., 2002; Mills and Chichester, 2005; Vajda et al., 2008). A USA nation-wide study of intersex was conducted by Hinck et al. (2009) in which researchers assessed the intersex condition in black basses at 111 sites in nine river basins. Their study found that the highest incidence of intersex within the USA occurred in the Yadkin-Pee Dee River basin of North Carolina and South Carolina. Another study, by Sackett et al. (2015), identified sites that were associated with point source contaminants (EACs), and these were sites where Hinck et al. (2009) found high occurrence of intersex. These previous studies provided the motivation to undertake an investigation into high incidences of fish intersex in the Yadkin-Pee Dee River and to examine their potential relationships to EACs. The specific objectives of our research were to (1) determine intersex occurrence and severity in common sport fishes in a large, regulated, southeastern USA river, (2) measure and evaluate contaminants in surface water and sediments of the same river, (3) investigate relationships between fish intersex and EACs, and (4) determine trends in EAC concentrations, intersex occurrence, and intersex severity, longitudinally within the Yadkin-Pee Dee River system. The ultimate goal of this research was to enhance the understanding of the relationship between contaminants and fish health to facilitate ecologically comprehensive conservation and management decisions.

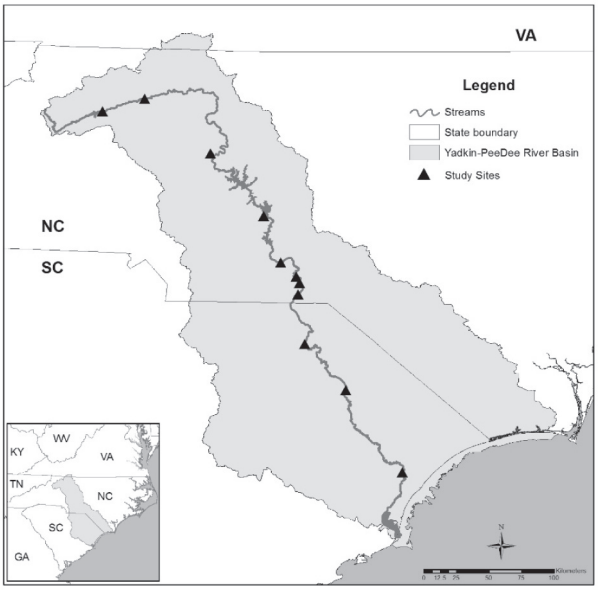
2. Methods

2.1. Study system

The Yadkin-Pee Dee River of North Carolina and South Carolina was chosen as our study system. A total of 11 sites were selected longitudinally along the river in North Carolina (8 sites) and South Carolina (3 sites) and site coordinates and descriptions are included in Fig. 1. Sites exhibited varying levels of anthropogenic influence, land use, and habitat types and were also selected for ease of boat access and sampling logistics. Eight sites were located in riverine habitats and three were located in reservoirs impounded by high dams. Three of the sites we studied (74 Bridge, Pee Dee, Bucksport) were sampled previously by Hinck et al. (2009) as sites PRB 336, 337, and 338.

2.2. Fish collection and histopathology

In April and May 2014, boat-mounted electrofishing (pulsed direct current) was conducted to capture wild, adult fish at all sites (within 2 km upstream or downstream of a river site or within the sampled reservoir). Additional sampling was conducted during June 2015 at the Bucksport, South Carolina, site because of the high incidence of intersex observed by Hinck et al. (2009) at this site. Collections from 2014 and 2015 catches from the Bucksport site were combined for analyses. Up to 10 male black bass (*Micropterus* spp.), 10 male sunfish (*Lepomis* spp.), and 10 male catfish (Ictaluridae) were collected at each site whenever possible. These taxa represent fish with varying life history strategies and are recreationally important sport fish within the river. Black bass have special relevance because of previous research conducted on both Largemouth Bass (*Micropterus salmoides*) and Smallmouth Bass (*Micropterus dolomieu*) examining intersex occurrence (Blazer et al., 2014; Hinck et al., 2009; Yonkos et al., 2014). Fish were collected at sizes indicative of sexual maturity, and any obvious females (eggs apparent when pressure was applied to the abdomen) were released back into the river. Euthanasia of male fish was completed following NC State University approved IACUC guidelines, with a lethal overdose of pH-buffered tricaine methanosulfate (MS-222,



Site	Latitude	Longitude	Type	State
Kerr Scott Reservoir	36.130355	-81.229061	Reservoir	NC
Ronda	36.21601	-80.937164	River	NC
Route 801	35.838287	-80.484752	River	NC
Badin Lake	35.407817	-80.114984	Reservoir	NC
Red Hill	35.0847222	-79.9986111	River	NC
Blewett Lake	34.9877778	-79.8911111	Reservoir	NC
74 Bridge	34.9455556	-79.8691667	River	NC
Diggs Tract	34.8652778	-79.8791667	River	NC
Society Hill	34.524154	-79.832815	River	SC
Pee Dee	34.203845	-79.547676	River	SC
Bucksport	33.661734	-79.153058	River	SC

Fig. 1. Location of the Yadkin-Pee Dee River basin in North Carolina and South Carolina and the 11 study sites, listed in order from upstream to downstream with their corresponding coordinates, site type and state of occurrence.

Sigma Aldrich). Sacrificed fish for tissue analyses (i.e., contaminant concentrations and histopathology) were selected randomly among those captured of the target species, sex, and size. Length (total length, mm) and weight (g) of each individual were measured, and relative weight (W_r), an index of individual fish condition, was determined for each individual according to the formula (Neumann et al., 2012)

$$W_r = \left(\frac{body\ weight}{10^a \times length\ (mm)^b}\right) \times 100.$$

Intercept (a) and slope (b) parameters for each species are in Table SI 1. Following measurements, sex was verified macroscopically and male internal organs were removed. Testes were weighed and gonadosomatic index (GSI; Strange, 1996) was calculated as

$$GSI = \left(\frac{gonad\ weight}{body\ weight}\right) \times 100.$$

Liver was removed, weighed, and hepatosomatic index (HSI; Tveranger, 1985) was calculated as

$$HSI = \left(\frac{liver\ weight}{body\ weight}\right) \times 100.$$

Fish liver and testes were preserved in modified Davidson's fixative (35.1% distilled water, 31.4% ethanol, 22.0% formalin [37–40% formaldehyde], and 11.5% glacial acetic acid) for histological analysis and to measure liver weight. Fish carcasses were placed in food-grade, zip-sealable, plastic bags, labeled, and stored on ice until frozen at −20 °C for later muscle tissue dissection. Excised organs were left in fixative for 24 h and then transferred to 70% ethanol until histological processing occurred. Testes were either embedded whole (for small fish specimens) or representative cross sections were placed into histological processing cassettes. Fixed tissues were routinely processed by the North Carolina State University (NCSU) College of Veterinary Medicine Histopathology Laboratory (Raleigh, North Carolina), embedded in paraffin, sectioned at 5 μm, and stained using hematoxylin and eosin. Following histological preparation, a board certified (American College of Veterinary Pathologists) fish pathologist (JML) examined testis tissue slides using light microscopy for the occurrence and severity of intersex, which for this study was defined as the presence of oocytes within the testis tissue. Intersex severity was determined using the method developed by Blazer et al. (2007), where severity is ranked 1 to 4, with 4 being the most severe ranking. Whenever possible, 5 cross-sections of testis were examined from each fish as prescribed by Blazer et al. (2007). In the case of smaller fish, however, testes were embedded whole in longitudinal orientation and only 3 sections were available for analysis; nonetheless, intersex severity rank was still able to be determined.

2.3. Water estrogenic activity evaluation

Water samples were collected using solvent-rinsed and baked 2-L amber glass bottles. At each site, a subsurface sample was collected and acidified to a pH of 2. Samples were placed on ice and transported to NCSU where they were stored at 4 °C for a maximum of 72 h before processing. Water samples were then filtered and solid phase extraction was completed. Extracts were analyzed for total estrogenic activity by a T47D-Kbluc bioassay, which uses human breast adenocarcinoma cells and a 17β-estradiol (E2β) standard to determine E2β equivalent concentrations (E2β Eq-ng/L). The bioassay and extraction process were detailed by Lee Pow (2015) and Yost (2014).

2.4. Muscle tissue: metal analysis and organic contaminant extraction

A subsample of fish, representing all of the sites, major genera, and adult fish sizes was selected for muscle tissue contaminant analyses. Muscle tissue was selected for contaminant analyses to represent the consumption load and human health risk of anglers that harvest and consume these sport fish species. Composite samples of individuals of the same species and site were made when adequate tissue from an individual was not available (this was only necessary for sunfish). Using standard fish processing protocols, muscle tissue samples were dissected and homogenized (US EPA, 2000). Following homogenization, samples were frozen at −80 °C until further processing. In preparation for contaminant analyses, muscle tissue samples were lyophilized and manually homogenized. RTI International (Durham, North Carolina) analyzed the fish tissue samples for 22 metals (Table SI 2). Mercury (Hg) concentration in tissue samples was determined using a Milestone

DMA-80 direct mercury analyzer. Other metals were analyzed with a modified version of Method 3050B (US EPA, 1996) and a Thermo X-Series II ICP-MS or a Thermo iCAP6500 ICP-OES depending on the concentration of the analyte present in the sample. The Analytical Toxicology Laboratory at NCSU (Raleigh, North Carolina) analyzed fish samples for PCBs and OCPs (Table SI 2) and determined percentage of lipids in each muscle tissue sample. For organics, lyophilized muscle tissue was extracted with dichloromethane (DCM) by means of pressurized solvent extraction using a Buchi Speed Extractor E-916. Extracts were then cleaned using gel permeation chromatography (GPC) and were processed through Florisil solid phase extraction cartridges.

2.5. Sediment: metal analysis and organic contaminant extraction

Sediment samples were collected at each site during May and June 2014. Samples were taken by wading from the shoreline in an upstream direction from the boat access. A composite sample, consisting of 6–10 grabs of surficial (top 5 cm) sediment, was collected using a stainless steel scoop and tray and placed into 250-mL amber glass jars for organic and metal contaminant analyses. Sediment was transported on ice and stored at −20 °C until extraction. RTI International (Durham, North Carolina) analyzed sediment samples for 22 metals (Table SI 2) following the protocol described above (section 2.4). The NCSU Analytical Toxicology Laboratory determined the concentrations of CUPs, OCPs, PAHs, and PCBs (Table SI 3). Sediment samples were extracted with DCM by means of pressurized fluid extraction using a Buchi Speed Extractor E-916. Extracts were cleaned using GPC, and before chemical analysis, extracts were concentrated to 0.5 mL under a gentle stream of nitrogen.

2.6. Water: organic contaminant extraction

Waterborne organic contaminants were measured using passive sampling devices (PSDs; Heltsley et al., 2005; O'Neal, 2014). PSDs were deployed at each site for approximately 28 days to determine time-weighted estimated concentrations of waterborne contaminants. Two types of PSDs were deployed. Low-density polyethylene strips (PEPSD) were utilized to measure OCPs, PCBs, and PAHs, and a sorbent-containing cartridge universal passive sampling device (UPSD) was used to assess CUPs, hormones, and industrial EACs (Table SI 3). A weighted cage containing both types of PSDs was connected to the riverbank upstream and on the opposite bank of the boat access (to reduce impacts directly from boaters and deter vandalism of cages) or at least 100 m away from the boat access in reservoirs. Each cage was suspended in the water column by a buoy and kept from moving downstream by attaching a brick. After approximately 28 days, PSDs were retrieved from each site, removed from their cages, wrapped in baked aluminum foil, placed into food-grade plastic bags, held on ice, transported to NCSU, and stored at −20 °C until extraction. PEPSDs were serially extracted three times over a 24-h period with a total of 150 mL of DCM. UPSDs analyzed for CUPs were placed in 20-mL vials and serially extracted two times over a 4-h period with a total of 40 mL DCM. Extracts were concentrated, filtered, and stored at −20 °C until analysis. Extracts were further concentrated under a gentle stream of nitrogen to approximately 0.5 mL just prior to analysis. UPSDs analyzed for hormones and industrial EACs were placed in 20-mL vials and extracted with 10 mL of ethyl acetate by shaking for 1 h at 150 rpm. Nitrogen evaporation at 35 °C under 34.5 kPa was then conducted to reduce extracts to 0.5 mL. Extracts for hormone and industrial EAC analysis were additionally filtered through a 0.45-μm polytetrafluoroethylene filter into a 1.5-mL microvial, evaporated with a nitrogen stream at ambient temperature until dry, and cap-sealed with argon gas. Complete description of PSD protocols, extraction procedures, and analytical instrumentation are detailed by O'Neal (2014) and Lee Pow (2015).

2.7. Analysis of sediment, water, and fish muscle extracts

Extracts from PSDs and sediment samples were analyzed for 42 PAHs, 28 OCPs, 21 PCBs, and 47 CUPs. PSD extracts were also analyzed for concentrations of 7 water-borne estrogen-related hormones and 2 industrial EACs. Fish muscle tissue extracts were analyzed for 21 PCBs and 28 OCPs (Table SI 2). CUPs, OCPs, PAHs, and PCBs were measured using an Agilent 6890 gas chromatograph (GC) connected to an Agilent 5973 mass selective detector (MSD) operated in Select Ion Monitoring (SIM) mode. Analytes were separated on a Restek Rtx-5MS column with a 5 m integrated guard column. Hormones and industrial EACs were analyzed on an Agilent 7890 GC connected to an Agilent 7000 MSD operated in SIM mode, with back flushing following a blank injection of pyridine to condition the column. All analyses adhered to rigorous quality assurance protocols and included procedural blanks, replicate samples, spiked samples, and data correction using surrogate recoveries, if necessary. Water contaminant results are presented in ng/L, and sediment and muscle tissue contaminant results are presented in ng/g dry weight (DW). Detection limits (DL) for waterborne contaminants were 0.2 ng/L for PAHs, PCBs, and OCPs, 0.5 ng/L for CUPs, and 0.1 ng/L for hormones, nonylphenol, and BPA (based on an equivalent 28-day PSD deployment). DLs for sediment and muscle tissue contaminants were 0.1 ng/g for OCPs and PCBs, 1.0 ng/g for PAHs, and 2.0 ng/g for CUPs (all DW). Sediment DLs, for metals that were detected, are listed in Table SI 4. DLs for metals in muscle tissue are identical to the DLs for sediment. Muscle tissue results, when necessary for comparisons with thresholds, are presented in wet weight by conversions using percent moisture in each sample.

Chemicals were evaluated both as individual analytes and as total concentrations of chemical classes, when appropriate. For PAHs, individual analytes were examined, and a total sum of analytes was determined to compare to predicted effect concentrations and threshold effect concentrations (PECs; TECs; MacDonald et al., 2000). PAHs in water and sediment samples were also analyzed using the equilibrium partitioning sediment benchmark toxicity units (ESBTU) method (US EPA, 2003), which incorporates the additive nature of PAH toxicity and the bioavailability of PAHs due to organic carbon in the sediment. Of the 42 PAHs analyzed, 34 have PAH potency divisors (unique for acute or chronic exposures) published by the US EPA (2003). Using the acute or chronic potency divisor, the determined concentration and, for the sediment sample, the carbon content of the sediment sample, a toxic unit (TU) was determined for each PAH (acute and chronic values). Then, all 34 PAH toxic units were summed to determine an overall PAH toxic unit value, with a value <1.0 indicating that it is unlikely for the PAHs to cause adverse effects to aquatic life, and a value >1.0 indicating that aquatic life is possibly being negatively affected.

2.8. Statistical analysis

All statistical analyses were completed using JMP Pro 12 (SAS, Cary, North Carolina). The Shapiro-Wilk normality test was conducted on all continuous variables to assess normality. The majority (>50%) of variables did not conform to a normal distribution, and the condition was not remedied by transformation in most variables; thus, non-parametric statistical procedures were applied. Intersex occurrence is presented as a percentage of fish sampled at each site with the intersex condition, and intersex severity is presented as the mean severity index (1–4) among intersex individuals at each site. A Kruskal-Wallis test was applied to assess relationships between species and fish health parameters, intersex severity ranking/occurrence and genera, intersex condition and fish health parameters, and fish families and muscle tissue contaminants. Linear regression was utilized to determine relationships between environmental concentrations of contaminants and fish muscle tissue contaminant concentrations. Logistic regression was performed to explain intersex condition and severity index of those with intersex by fish muscle tissue contaminant concentrations in individual black bass (the only genus with sufficient intersex occurrence);

response variables were the presence (or absence) of intersex (categorical) and severity index (ordinal). Principal component analysis (PCA) was used to assess the trends among environmental contaminants, intersex occurrence, and intersex severity within the river. Analyses were weighted by the number of individuals collected at each site. All significance levels were set to $\alpha = 0.05$.

We adopted an information theoretic approach to model contaminant–intersex relationships. All subsets regression was performed to identify plausible relationships between environmental contaminants and intersex occurrence and severity. The candidate model included waterborne and sediment contaminant concentrations. The sum of chemical contaminants within a class, or a representative contaminant from each class, was selected as independent variables for model development. Atrazine (representative CUP, water, ng/L), industrial EACs (sum of BPA and nonylphenol, water, ng/L), ethinylestradiol (EE2, water, ng/L), PCBs (sum of all congeners, water, ng/L), OCPs (sum of all OCPs, water, ng/L), PAHs (sum of all PAHs, water, ng/L), PAHs (sum of all PAHs, sediment, ug/g DW), OCPs (sum of all OCPs, sediment, ug/g DW), and Hg (representative metal, sediment, ug/g DW) were the predictors selected based on their occurrence in the river, co-linearity, environmental relevance, and background knowledge of EACs and their influence on intersex in fish. If a contaminant concentration was not detected, the value used for statistical procedures was expressed as 50% of the DL. Akaike's information criteria corrected for small-sample bias (AICc) was determined for each candidate model (Akaike, 1973; Burnham and Anderson, 2002). Candidate models were arranged from lowest to highest AICc, and a ΔAICc was calculated for each model. Akaike weight (w_i) was calculated for each model to determine the relative likelihood of each model, with the highest w_i indicating the most plausible model (Burnham and Anderson, 2002). Then, a set of confidence models was identified with w_i within 10% of the best-model w_i to evaluate model strength (Royall, 1997; Ruiz and Peterson, 2007; Thompson and Lee, 2000). To determine relative variable importance, variables were ranked by summing all w_i values of models that included a specific variable. Total w_i for variables were compared and ranked, and the variable with the highest w_i was deemed the greatest importance. The all subsets regression modeling process was completed for black bass and sunfish intersex occurrence and average severity.

3. Results

3.1. Fish health parameters

In 2014 and 2015, we collected a total of 268 male black bass, sunfish, and catfish ($n = 80$, $n = 112$, $n = 76$; respectively, 12 species total). Species and the number of individuals collected at each site varied (Table 1). Individuals from each genus and family were captured at each site except for the Route 801 site, where only Ictaluridae were collected. Largemouth Bass, Bluegill (*Lepomis macrochirus*), and Blue Catfish (*Ictalurus furcatus*), were collected most frequently in the black bass, sunfish, and catfish groups, respectively. Seventy-eight individual fish were collected from reservoirs and 190 from riverine sites. No significant difference ($p > 0.05$) was detected in the length or weight among the different black bass species when pooling all individuals from all sites (Fig. SI 1A, B). GSI, HSI, and W_r values were significantly different among black bass species ($p < 0.05$; Fig. SI 1C, D, E). Length, weight, GSI, and W_r were significantly different among sunfish species ($p < 0.05$; Fig. SI 1A, B, C, E), but no significant difference in HSI was detected (Fig. SI 1D). Length, weight, HSI, and W_r differed among catfish species, but GSI was not significantly different among catfish species (Fig. SI 1A, B, C, D, E).

3.2. Fish intersex occurrence and severity

Intersex was observed in 40% of black bass (32 of 80 collected), 7% of sunfish (8 of 112 collected), and 1% of catfish (1 of 76 collected). Specific intersex occurrence for each species at each site is found in Table 1. Intersex occurrence in black bass ranged 6.7–100.0% among the 10 sites where they were collected (Fig. 2A). Average severity of black bass exhibiting intersex was 2.7. Sunfish intersex occurrence ranged 0.0–16.7% among the 10 sites from which they were collected (Fig. 2B). Average severity of intersex sunfish was 1.8 (Table 1). Intersex in catfish was only observed at the most downstream site (Bucksport, SC), with only one individual displaying the condition (7.1% occurrence, severity 2; Fig. 2C; Table 1). Due to the low occurrence of catfish with the intersex condition, they were excluded from further statistical analyses. Intersex occurrence and fish health parameters were only compared for black bass because they were the only fish group with sufficient sample sizes of both intersex and non-intersex individuals. When comparing black bass length (mm), weight (g), W_r, GSI, and HSI between intersex and non-intersex individuals, there were no significant differences ($p > 0.05$). There were also no differences in these fish parameters among the severity ranking groups ($p > 0.05$).

3.3. Muscle tissue contaminants

A total of 30 black bass, 28 sunfish (14 composite samples), and 29 catfish muscle tissue samples were analyzed for organic and inorganic contaminants, and results for each species at each site can be found in Table SI 5. Black bass samples had a mean percent moisture of 79.8%,

Table 1
Fish collected at each site in the Yadkin-Pee Dee River of North Carolina and South Carolina, listed in order from upstream to downstream and by fish species and group. Numbers in parentheses are the number of individuals with intersex and the mean intersex severity index. No number within parentheses indicates that intersex was not detected. LMB: Largemouth Bass; SMB: Smallmouth Bass; SPB: Spotted Bass; RES: Redear Sunfish; WAR: Warmouth; BLG: Bluegill; RBS: Redbreast Sunfish; PKS: Pumpkinseed; CHC: Channel Catfish; WHC: White Catfish; BLC: Blue Catfish; FHC: Flathead Catfish.

	Black bass			Sunfish					Catfish			
Site	LMB	SMB	SPB	RES	WAR	BLG	RBS	PKS	CHC	WHC	BLC	FHC
Kerr Scott R.	3		7 (1,4)	1	2	10 (1,1)	1		1	6		
Ronda		2	5 (1,2)			1	4		1			
Route 801									1		2	1
Badin Lake	10 (8, 2.6)			3 (1,2)		9	1			2		
Red Hill	2 (2,3)			2		8			2		4	1
Blewett Lake	10 (2,2)					10			2			
74 Bridge	3 (1,4)			2			8 (1,1)		5		5	
Digg's Tract	5 (4,2.3)			4		4	4 (2,2)		6		4	
Society Hill	8 (6,3.7)			1 (1,4)		9 (1,1)			2		6	2
Pee Dee	10 (6,2.3)			2		8			1		5	3
Bucksport	15 (1,1)			9 (1,1)	1	6	1	1			11 (1,2)	3
Total collected per species	66	2	12	24	3	65	19	1	21	8	37	10
Total collected per group	80			112					76			
Intersex per group(%)	40.0 ($n = 32$)			7.1 ($n = 8$)					1.0 ($n = 1$)			
Mean severity per group	2.72			1.75					2			

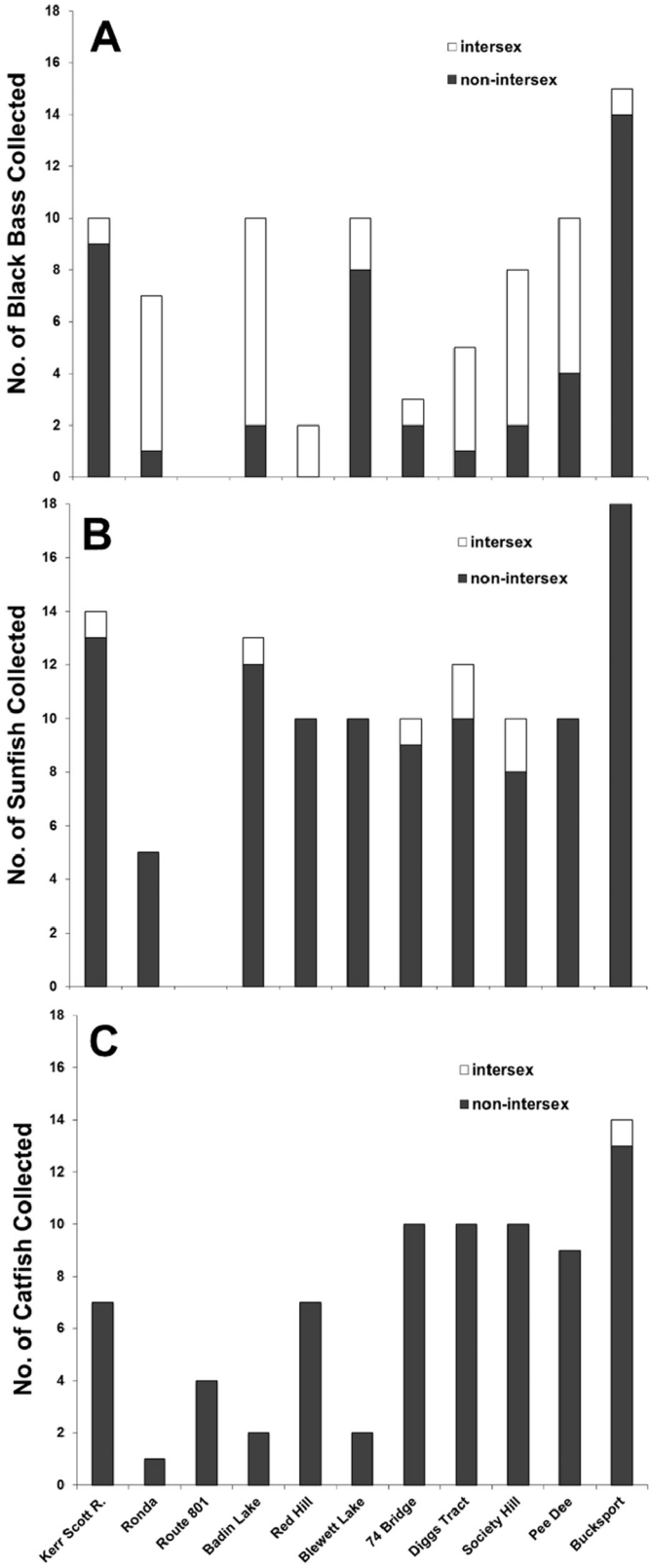


Fig. 2. Total number of black bass (A), sunfish (B), and catfish (C) analyzed for intersex at each site. Sites are arranged (left to right) from upstream to downstream.

catfish 78.6%, and sunfish 80.0%. OCPs were present in the muscle tissue of all three taxa, with 6 of the 28 OCPs detected. OCPs detected in black bass tissue were hexachlorobenzene, trans-chlordane, cis-chlordane, trans-nonachlor, 4,4′-DDE, and 4,4′-DDD (21% of the 28 OCPs analyzed). Hexachlorobenzene and 4,4′-DDD were present in only 1 and 2 samples, respectively. Total OCP concentration (sum of all OCPs detected) in black bass tissue samples ranged 2.2–88.0 ng/g dry weight (DW) (0.6–18.4 ng/g wet weight (WW)). OCPs detected in sunfish were heptachlor epoxide, trans-chlordane, cis-chlordane, trans-nonachlor, and 4,4′-DDE (18% of the 28 OCPs analyzed). Heptachlor epoxide was detected in only one sample. OCP concentrations in sunfish ranged from below detection limits (BDL) to 27.1 ng/g DW (BDL–5.2 ng/g WW). Trans-chlordane, cis-chlordane, trans-nonachlor, 4,4′-DDE, and 4,4′-DDD were OCPs detected in catfish muscle tissue. 4,4′-DDD was detected in only four samples. Concentrations of OCPs in catfish ranged BDL–127.6 ng/g DW (BDL–39.1 ng/g WW). OCP concentrations among the black bass, sunfish, and catfish were significantly different ($p < 0.0002$), with catfish and black bass having higher concentrations of OCPs than sunfish (Fig. 3A). No significant differences were detected in OCP concentrations within species of black bass, sunfish, or catfish when individuals from all sites were pooled ($p > 0.05$). OCP concentrations in fish tissue varied among sites, with highest OCP tissue concentrations occurring at the 74 Bridge, Digg's Tract, Badin Lake, and Route 801 sites (Fig. 4A). There was a significant difference ($p = 0.0007$) among sites (with all species pooled) with the Route 801 site having higher concentrations of OCPs than the rest of the sites (Fig. 4A). This significant difference, however, is most likely influenced by low sample size ($n = 2$) and because only catfish were collected at this site.

Fish muscle tissue samples were analyzed for 21 PCB congeners, and 14 (67%) of those congeners were detected (Table SI 2). Concentrations of individual PCB congeners were summed as a total PCB concentration for each muscle tissue sample. Total PCB concentration in black bass ranged 1.37–202.40 ng/g DW (0.31–42.5 ng/g WW). In sunfish samples, it ranged BDL–42.3 ng/g DW (BDL–12.8 ng/g WW), and in catfish total PCB concentration ranged 4.6–215.2 ng/g DW (0.89–65.9 ng/g WW). PCB concentrations were different among black bass, sunfish, and catfish ($p < 0.0001$; Fig. 3B) with significantly lower PCB concentrations in sunfish. Significant differences in PCB concentrations were detected

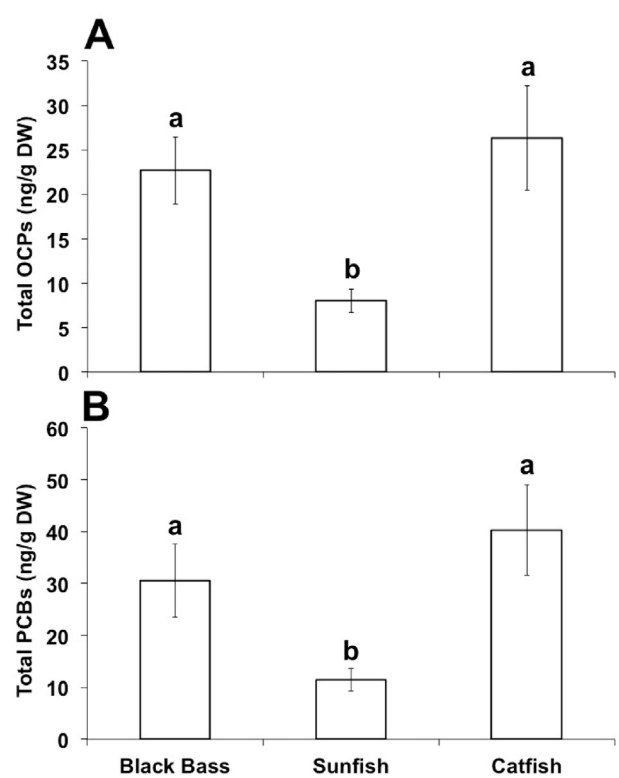


Fig. 3. Total OCP (A) and total PCB (B) concentration in black bass, sunfish, and catfish muscle tissue (mean ± standard error). Sites having the same letter indicate no significant difference ($p > 0.05$).

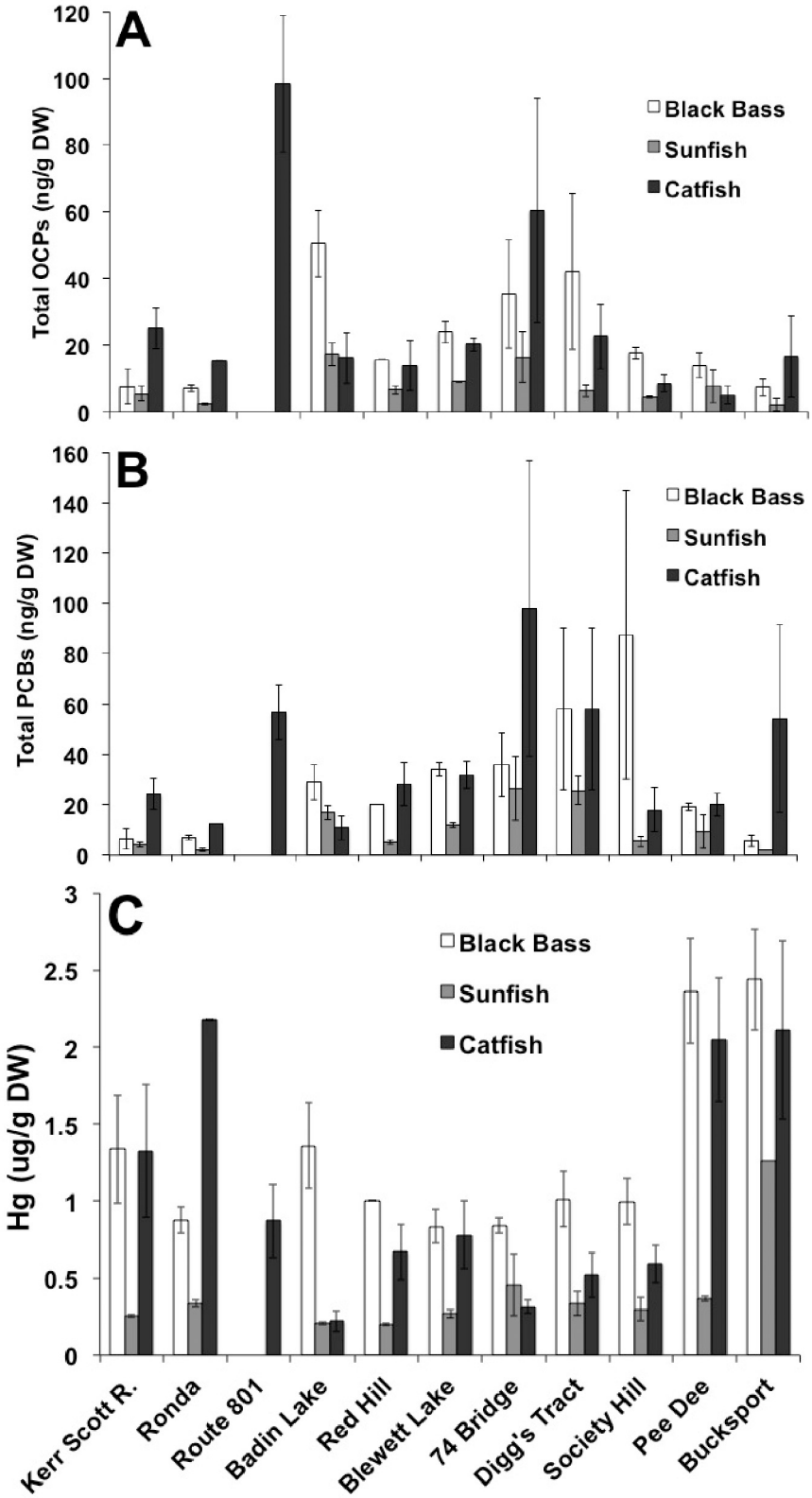


Fig. 4. Total OCP (A), total PCB (B), and Hg (C) muscle tissue concentrations at each site (mean ± standard deviation).

among black bass species ($p = 0.029$) and catfish species ($p = 0.045$); however, some sample sizes were small ($n \leq 5$). No significant differences were detected in PCB concentration among sunfish. Among sites, average PCB concentrations (DW of black bass, sunfish, and catfish) varied and were highest at the 74 Bridge, Digg's Tract, and Society Hill sites (Fig. 4B). No significant differences were detected in PCBs, with all species pooled among sites ($p > 0.05$). Among all fish tissue sampled, the North Carolina Department of Public Health total PCB action level for human consumption of freshwater fish, 50 ng/g WW, was exceeded in one catfish, at the 74 Bridge site (NC DPH, 2007).

Metal analysis revealed that 14 of the 22 metals were predominant (above DL in at least 20% of samples) in black bass, sunfish, and catfish tissue (Table SI 2). These metals included aluminum (Al), barium (Ba), copper (Cu), iron (Fe), mercury (Hg), potassium (K), magnesium (Mg), manganese (Mn), nickel (Ni), lead (Pb), selenium (Se), silicon (Si), strontium (Sr), and zinc (Zn). The only metal detected in fish tissue that was of concern (greater than the human health consumption advisory and/or aquatic wildlife thresholds, when available) was Hg (Fig. 4C; NC human health consumption threshold = 400 ng/g, muscle tissue WW). Concentrations of Hg were highest (exceeded 400 ng/g WW) in black bass and catfish at the two most downstream sites, Pee Dee and Bucksport. Hg muscle tissue concentration was significantly lower in sunfish when compared to black bass and catfish.

3.4. Water E2β equivalence and contaminants

E2β equivalence was determined for each of the 11 sites (one sample per site), and estrogenicity was detected at every site. Concentrations ranged 0.10–1.26 E2β Eq-ng/L (Fig. 5A; Table SI 3). E2β equivalent concentrations exceeded the 0.73 ng/L predicted-no-effect concentration (PNEC) for E2β at two sites, Route 801 and 74 Bridge (Wu et al., 2014).

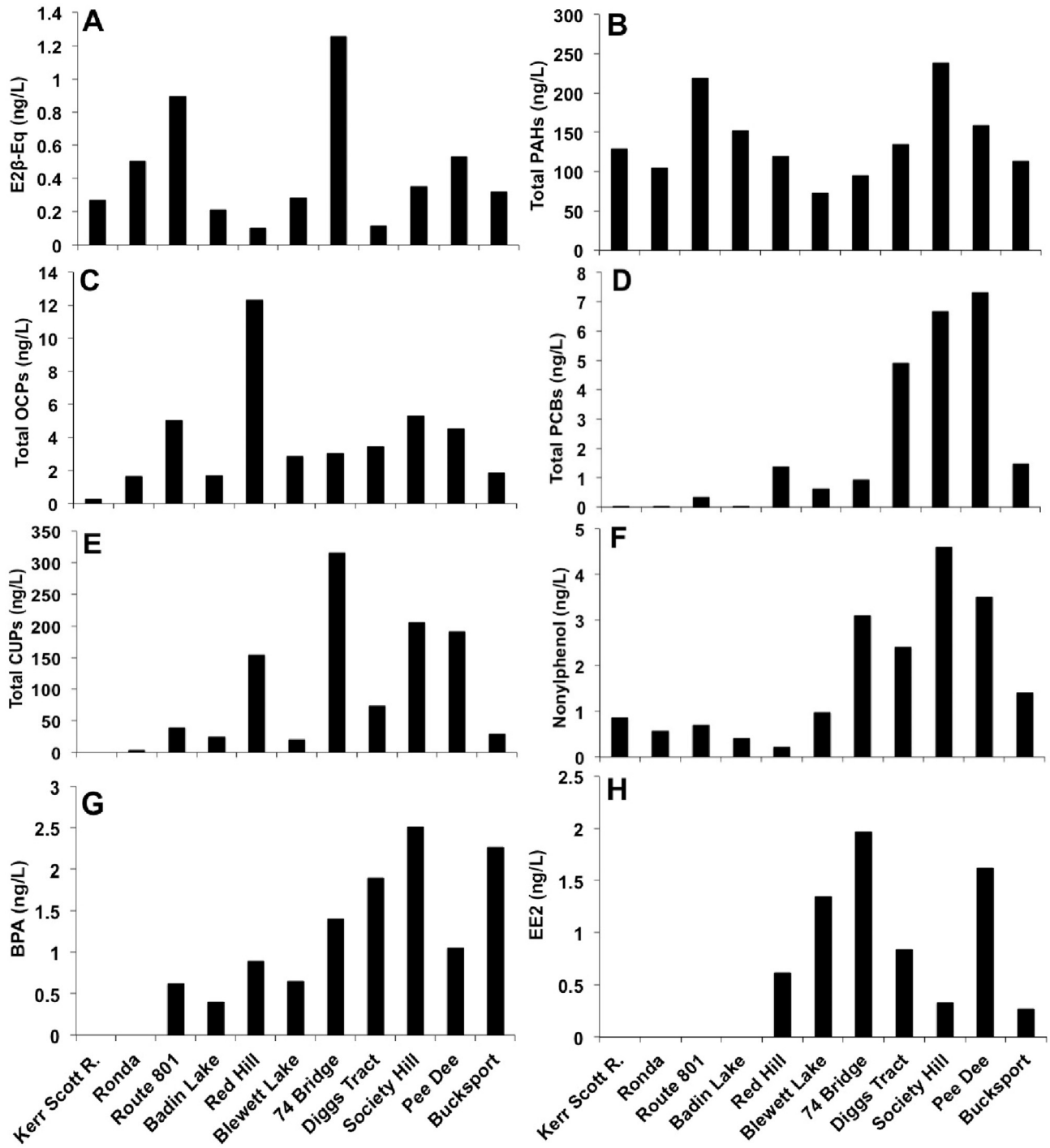


Fig. 5. Concentrations of E2β-Eq. (A), PAHs (B), OCPs (C), PCBs (D), CUPs (E), nonylphenol (F), BPA (G), and EE2 (H) in ng/L at each site.

PAHs were present in the water at 100% of the sites, and 33 of the 42 PAHs measured were detected (Table SI 2). PAHs were summed, and a total PAH value was determined (ng/L) for each site. Total PAH concentrations ranged from 72.6 to 238.2 ng/L (Fig. 5B; Table SI 3). Concentrations fluctuated among sites with the highest levels occurring at the Route 801 and Society Hill sites. Downstream of these sites, concentrations seemed to attenuate (Fig. 5B). Specific PAHs and their concentrations varied throughout the system with no obvious spatial trend. The most commonly detected PAHs in water were components of coal tar, such as pyrene, anthracene, and fluroanthene. ESBTU values ranged 0.009–0.035 TU for acute toxicity, all below 1. For chronic toxicity, TUs ranged 0.017–0.071 TU, all below 1, indicating a low likelihood for negative effects of waterborne PAHs on aquatic life (Table SI 6). ESBTU values were calculated with the concentrations of the 42 PAHs evaluated.

OCPs were present in water at 100% of the sites, and 9 of the 28 OCPs measured were detected (Table SI 2). OCPs were summed to get a total OCP concentration (ng/L) for each site. OCP concentrations ranged 0.26–12.29 ng/L (Fig. 5C; Table SI 3). No individual OCP concentration exceeded published aquatic life protection thresholds (US EPA, 2016). No combined OCP concentration threshold or aquatic life thresholds exist. OCPs were notably elevated at the Red Hill, Route 801, and Society Hill sites (Fig. 5C).

PCB congeners were present in water above detection limits at 8 of the 11 sites, and of the 21 PCB congeners evaluated, 10 were present in the river (Table SI 2). All PCB congeners were summed, and a total PCB concentration for each site was calculated (ng/L). Concentrations ranged BDL–7.31 ng/L (Fig. 5D; Table SI 3). Total PCB concentrations did not exceed the 14.0 ng/L chronic exposure threshold for freshwater aquatic life at any of the sites (US EPA, 2016). Concentrations were low (<1.5 ng/L) at all sites upstream of Digg's Tract and the most downstream site, Bucksport, but they were elevated (4.5–7.5 ng/L) at the Digg's Tract, Society Hill, and Pee Dee sites (Fig. 5D). Specific PCB congeners were more frequently detected and at higher concentrations at downstream sites; congeners 138, 153, 44, and 52 were most frequently detected.

CUPs were present in water at 10 of the 11 sites, with no CUPs detected at Kerr Scott Reservoir, the most upstream site. Of the 47 CUPs evaluated, 8 were present in the river (Table SI 2). CUPs were summed to get a total CUP concentration (ng/L) for each site, which ranged from BDL to 315.6 ng/L (Fig. 5E; Table SI 3). CUP concentrations were low at the uppermost sites, variable at intermediate sites, and decreased at the most downstream site, Bucksport. The herbicides atrazine, simazine, and metolachlor were most frequently detected and were at the highest concentrations downstream of the Red Hill site.

Both of the two industrial EACs evaluated (nonylphenol and BPA) were detected in river water. Nonylphenol was detected at all 11 sites and ranged 0.40–4.60 ng/L (Fig. 5F; Table SI 3). Concentrations of nonylphenol were low (<1 ng/L) at upstream sites and higher (>1 ng/L) at all sites downstream of Badin Lake (Fig. 5F). None of the measured concentrations exceeded the 6600 ng/L chronic exposure threshold determined by the US EPA (2005). BPA was present at 9 of the 11 sites, with no detections at the two most upstream sites. Concentrations of BPA ranged BDL–2.27 ng/L (Fig. 5G; Table SI 3). Concentrations of BPA were similar to those of nonylphenol, with concentrations low upstream (<1 ng/L) and higher (>1 ng/L) at downstream sites below Blewett Lake (Fig. 5G). BPA concentrations did not exceed the 1500 ng/L (PNEC), set by the European Union (Aschberger et al., 2010) for the protection of freshwater aquatic species, at any site. EE2 was the only hormone detected and was above detection limits at 7 of 11 sites. Concentrations of EE2 ranged BDL–1.97 ng/L (Fig. 5H; Table SI 3), and at all sites where EE2 was detected, the concentrations exceeded the 0.1 ng/L PNEC for aquatic organisms (Caldwell et al., 2012). The DL for EE2 was 0.1 ng/L, so any sites without detections should not be considered absent of EE2, similar to all other BDL instances, and was supplemented with half of the DL for modeling analysis. EE2 was detected at the 7 most downstream sites (Fig. 5H).

3.5. Sediment contaminants

Of the 22 metals analyzed in sediment, 18 were detected (Table SI 2). Concentrations at each site and thresholds for each metal (when available) are listed in Table SI 4. Mn exceeded the lowest effect level of 460 μg/g DW at 5 of the 11 sites and exceeded the severe effect level of 1100 μg/g DW at 1 site, Digg's Tract (Persaud et al., 1993; Fig. 6A). Cd exceeded the TEC of 0.99 μg/g DW at all sites (Fig. 6B), but did not exceed the PEC of 4.98 μg/g DW at any site (MacDonald et al., 2000). No other metal concentrations exceeded known benchmarks.

Of the 28 OCPs evaluated, 5 were detected in sediment samples (Table SI 2). OCPs were summed to get a total OCP concentration (ng/g DW) at each site. OCP concentrations ranged from BDL–101.49 ng/g DW with concentrations highest at the Badin Lake site (36 times higher than any other site, Table SI 3, Fig. 7A). This high level of OCPs at the Badin Lake site was due to the detection of hexachlorobenzene, which was not detected at any other site and accounted for 100% of the OCP concentration. The measured concentration at that site (101.49 ng/g DW) was well below a sediment benchmark for protection of benthic communities (76,500 ng/g DW; US EPA, 1995), and in a study by Fuchsman et al. (1998), limited toxicity to benthic invertebrates was observed (i.e., no significant toxicity up to 60,000 ng/g normalized to 1% organic carbon). 4,4′-DDE was the most frequently detected OCP with detections at 6 of 11 sites. 4,4′-DDE levels did not exceed the threshold of 3.16 ng/g DW for freshwater ecosystems at any site (MacDonald et al., 2000). Chlordane was also detected at two sites (Route 801 and Digg's Tract) but was below the 4.5 ng/g DW interim sediment quality guideline that corresponds to threshold level effects,

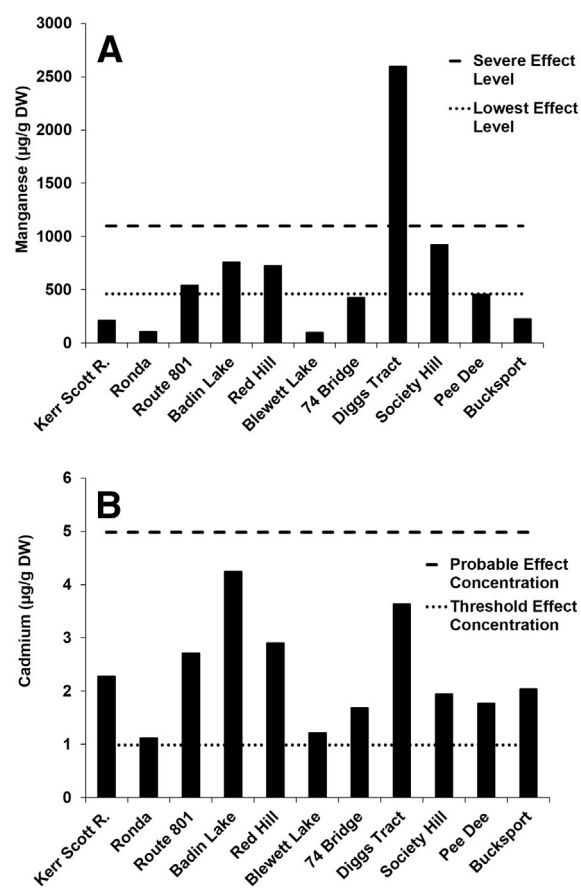


Fig. 6. Manganese (A) and cadmium (B) sediment concentrations at each site (μg/g DW), with severe effect and lowest effect manganese threshold level concentrations (Persaud and Jaagumagi, 1993) and probable effect (i.e., above which harmful effects are likely to be observed) and threshold effect (i.e., below which harmful effects are unlikely to be observed) cadmium concentrations (MacDonald et al., 2000).

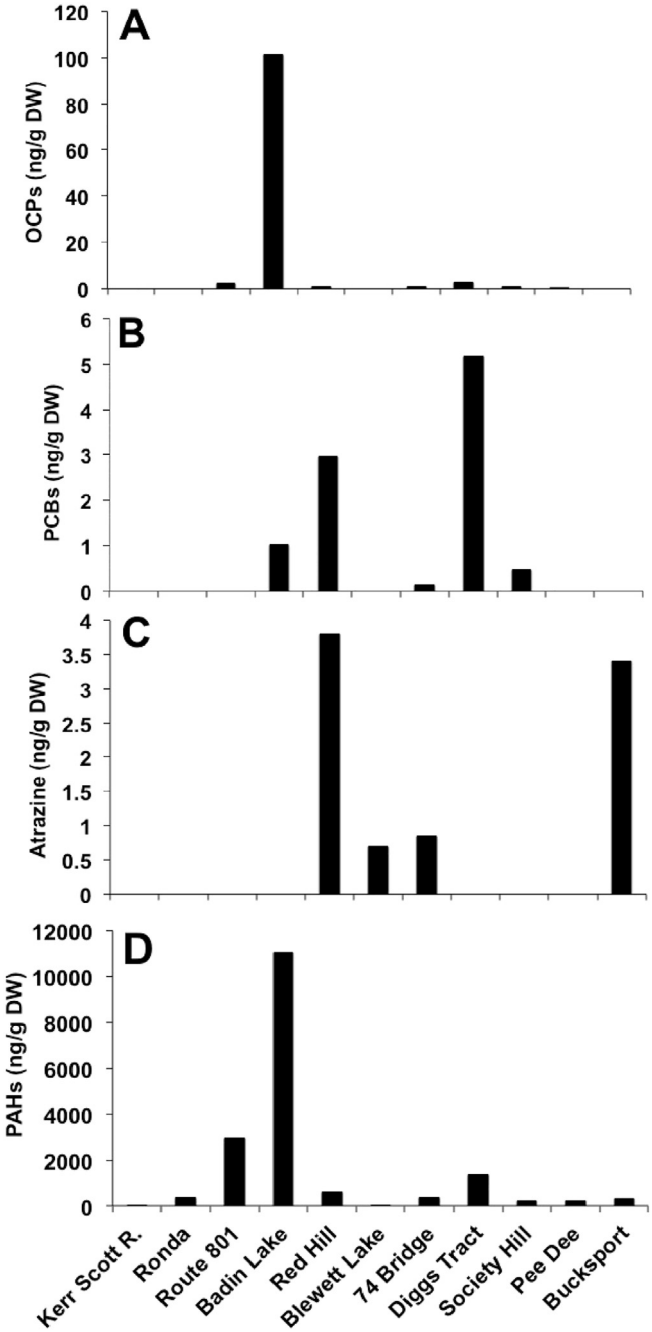


Fig. 7. Sediment concentrations of OCPs (A), PCBs (B), atrazine (C; the only CUP detected), and PAHs (D) in ng/g DW at each site.

below which adverse biological effects are not expected (Environment Canada, 1999).

PCBs were detected in sediment at only 5 sites and a maximum of 4 of 21 congeners were present at any site (Fig. 7B; Table SI 3). Overall, concentrations were low (<5 ng/g DW Total PCB concentration) and did not exceed the 59.8 ng/g DW threshold for freshwater ecosystems (MacDonald et al., 2000). Sites with PCB detections were Badin Lake and four more downstream sites (Fig. 7B). Atrazine was the only CUP detected in the sediment (Table SI 3). It was present at 4 of the sites and did not exceed the 6.62 ng/g DW screening benchmark developed by the US EPA (2006); Fig. 7C).

Thirty-nine of the 42 PAHs evaluated were detected in sediment (Table SI 2). PAHs were summed for a total PAH concentration in ng/g DW at each site. Total PAH concentrations ranged 1.66–11,068.05 ng/g DW (Fig. 7D; Table SI 3). PAH concentrations were similar at all sites except for Route 801, which was slightly elevated, and Badin Lake where PAH concentrations were eight times higher than average (Fig. 7D). Total PAH levels exceeded the 1610 ng/g DW TEC for freshwater ecosystems (MacDonald et al., 2000) at two sites, Route 801 and Badin Lake. PAH concentration did not exceed the PEC of 22,800 ng/g DW (MacDonald et al., 2000) at any site. ESBTU values ranged 0.0002–0.0690 for acute toxicity and 0.001–0.281 for chronic toxicity (Table SI 7). No sediment TUs exceeded 1, indicating little likelihood of sediment PAHs negatively affecting aquatic life.

3.6. Environmental contaminants and muscle tissue contaminants

We did not detect any statistically significant direct relationship between contaminant concentrations in the river environment and those in fish muscle tissue. PCB and OCP concentrations in the water were not significantly correlated to the muscle tissue concentrations of PCBs and OCPs found in black bass ($\rho < 0.6$). Sediment PCB and OCP concentrations were also unrelated to black bass PCB and OCP muscle tissue concentrations. There was no significant relationship between Hg in the sediment and black bass tissue. Sunfish muscle tissue contaminant concentrations were not significantly correlated to OCPs or PCBs in water, OCPs or PCBs in sediment, or sediment Hg. Catfish muscle tissue contaminant concentration was not significantly related to sediment Hg, PCBs, or OCPs. There was also no significant relationship between catfish muscle tissue PCBs or OCPs and PCBs or OCPs in water.

3.7. Intersex and contaminants

The percent occurrence of the intersex condition in black bass and sunfish was significantly correlated with metal and organic contaminant concentrations in the sediment among sites. Black bass intersex occurrence (% of fish at each site with the intersex condition) was significantly correlated with Cu (sediment, μg/g DW, Spearman's correlation coefficient, $\rho = 0.72$), Hg (sediment, μg/g DW, $\rho = 0.67$), Pb (sediment, μg/g DW, $\rho = 0.64$), Mn (sediment, μg/g DW, $\rho = 0.78$), Sr (sediment, μg/g DW, $\rho = 0.66$), PAHs (sediment, μg/g DW, $\rho = 0.64$), PCBs (sediment, μg/g DW, $\rho = 0.88$) and OCPs (sediment, μg/g DW, $\rho = 0.89$). Black bass intersex severity was not correlated to any environmental variable. Sunfish intersex occurrence was significantly correlated with Ba (sediment, μg/g DW, $\rho = 0.65$) and Si (sediment, μg/g DW, $\rho = 0.70$). Sunfish severity was correlated with Ba (sediment, μg/g DW, $\rho = 0.78$), Cu (sediment, μg/g DW, $\rho = 0.64$), Hg (sediment, ng/g DW, $\rho = 0.69$), Mn (sediment, μg/g DW, $\rho = 0.67$), Si (sediment, μg/g DW, $\rho = 0.83$), and Sr (sediment, μg/g DW, $\rho = 0.64$).

Prevalence of black bass intersex condition often was found to correlate with higher levels of contaminants. PAHs, OCPs, CUPs, and PCBs in water (ng/L) were significantly higher at sites where more black bass with the intersex condition were collected, relative to those where fewer fish with the condition were collected. PAHs, OCPs, and PCBs were significantly higher in the sediment (μg/g DW) at sites with more intersex black bass individuals, compared to sites with fewer intersex black bass. Ba, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Si, Sr, and V concentrations (sediment, ng/g DW) were also significantly higher at sites with intersex individuals. K and Mg (sediment, μg/g DW) were significantly lower at sites with intersex individuals.

Few of the measured contaminant concentrations in individual black bass muscle explained the occurrence of intersex in individual fish and the severity of those with the intersex condition. Intersex occurrence in black bass was significantly and positively explained only by Cu (ug/g, DW, muscle tissue, $p = 0.0459$). When assessing the relationship between muscle tissue contaminants and the intersex severity index of black bass, we found that total PCBs (ng/g DW, $p = 0.0056$), Al (ug/g, DW, $p = 0.0072$), Mn (ug/g, DW, $p = 0.0116$), and Se (ug/g, DW, $p = 0.0005$) were positively and significantly correlated to intersex severity.

3.8. Principal components analysis

Principal component analysis yielded two principal components that accounted for 68.9% of data variability for black bass intersex occurrence and severity (Fig. 8A, Table SI 8). Sites with high intersex occurrence and severity clustered most closely to PAHs in the water. Occurrence and severity were similarly related to the other waterborne and sediment contaminants. Sites with lower intersex occurrence (<20%) were clustered and minimally correlated with any EACs. Two principal components accounted for 63.2% of data variability in sunfish intersex occurrence and severity (Fig. 8B, Table SI 8). Occurrence closely clustered with PAHs in the water. Severity grouped evenly between Hg in the sediment and PAHs in the water. Lower (<8%) intersex occurrence sites clustered together, unassociated with EAC variables. Intersex occurrence and severity in sunfish and black bass both grouped between waterborne and sediment sequestered EACs, with PAHs in the water and Hg in the sediment most correlated for black bass and sunfish, respectively.

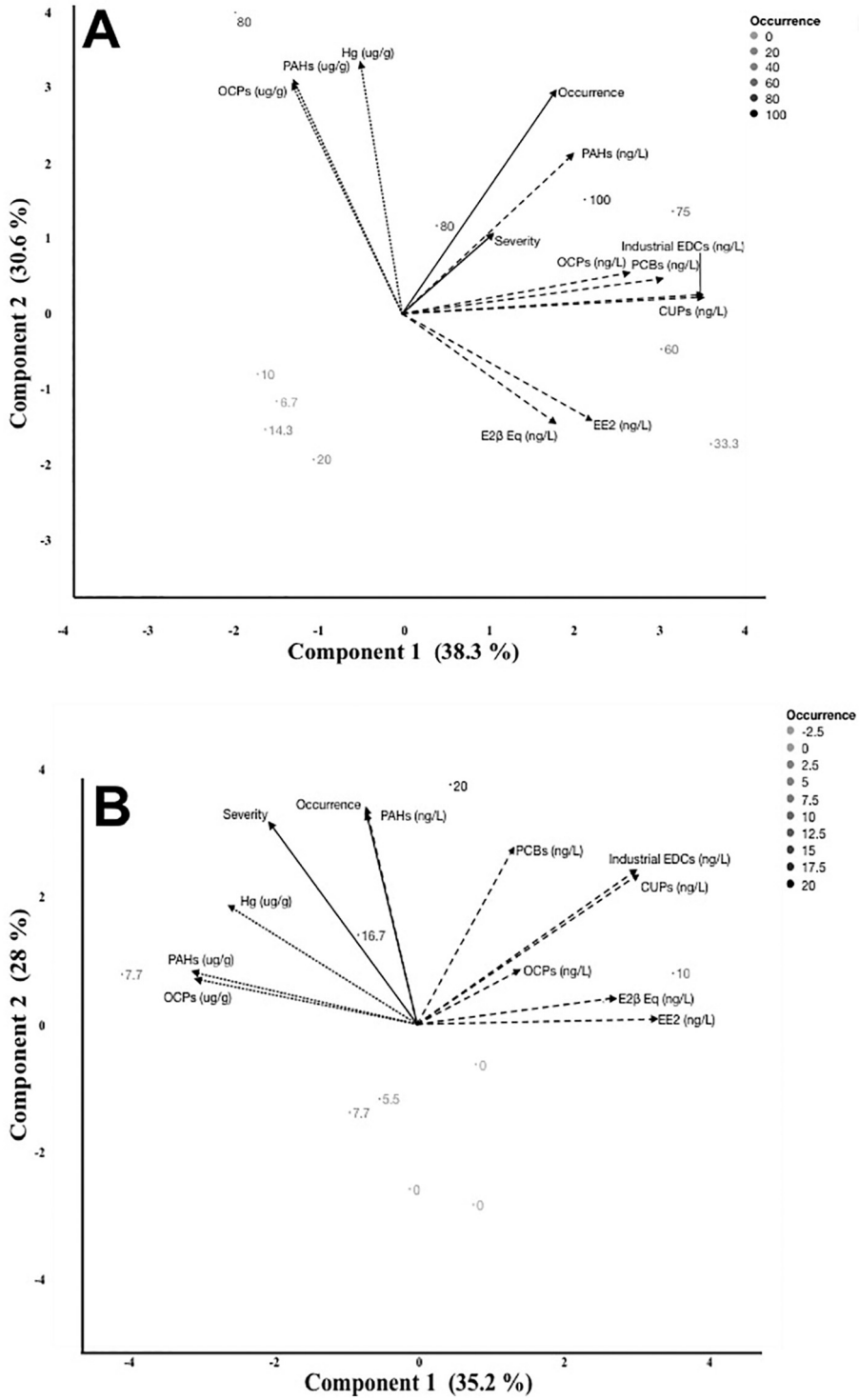


Fig. 8. Principal components analysis for black bass (A) and sunfish (B) intersex and severity (solid lines) with sediment PAHs, OCPs, and Hg (ug/g DW, dotted lines) and water CUPs, industrial EACs, PCBs, OCPs, EE2, PAHs, and E2β-Eq (ng/L, dashed lines). Labels are percent intersex occurrence per site (see legend).

3.9. Intersex model selection

A total of 10 confidence models were developed for bass intersex occurrence using the AIC_c analysis for model selection. The most plausible candidate model weight (w_i) was 0.244, and thus, candidate models with w_i >0.0244 (10% of the best-fitting model) were selected as confidence models (Table 2). Top confidence models included OCPs (water) and Hg (sediment) as explanatory variables. Variable ranking also identified OCPs (water) and Hg (sediment) in the top two rankings for the bass intersex occurrence model (Table 3). Bass intersex severity modeling yielded three confidence models. The most plausible candidate model weight was 0.4317, and two more models were selected within the 10% w_i range (Table 2). Only two variables were included within these three models, atrazine (water) and industrial EACs (water). These variables were also highly ranked in explaining black bass intersex severity (Table 3).

A total of five confidence models were developed for sunfish intersex occurrence. The most plausible model w_i was 0.3452, and four additional confidence models were selected with $w_i \geq 0.03452$ (Table 2). All confidence models included a single variable, with PAHs (water) and PCBs (water) being most plausible. PAHs (water) and Hg (sediment) were the highest-ranking model variables (Table 3). Sunfish intersex severity modeling yielded 10 confidence models. The most plausible model included PAHs (water, ng/L, $w_i = 0.2645$), and nine additional confidence models were selected with $w_i \geq 0.02645$ (Table 2). PAHs (water) and Hg (sediment) were the top ranking variables for this model set (Table 3).

4. Discussion

4.1. Intersex occurrence and severity

Our results indicate that the intersex condition occurs in fish throughout the Yadkin-Pee Dee River at varying frequencies, and that it is dependent upon species and related to EACs. Black bass displayed the intersex condition most frequently, which is similar to findings of other studies that examined intersex in riverine fishes (Lee Pow et al.,

Table 3
Rankings of contaminant concentration variables in water or sediment that explain among-site variance in fish intersex occurrence for black bass and sunfish in the Yadkin-Pee Dee River of North Carolina and South Carolina. Highest sum AICc weight (i.e., highest value) and rank (i.e., lowest number) variables are most explanatory.

Intersex	Taxon	Variable	Rank	Sum AICc Weight
Occurrence	Black bass	OCPs-water	1	0.7965
		Hg-sediment	2	0.6436
		PCBs-water	3	0.3896
		PAHs-sediment	4	0.2193
		OCPs-sediment	5	0.1027
		PAHs-water	6	0.0699
		Atrazine-water	7	0.0366
		EE2-water	8	0.0190
		Industrial EACs-water	9	0.0004
	Sunfish	PAHs-water	1	0.5167
		Hg-sediment	2	0.1364
		PCBs-water	3	0.1304
		Industrial EACs-water	4	0.1017
		Atrazine-water	5	0.0996
		OCPs-water	6	0.0894
		EE2-water	7	0.0839
		PAHs-sediment	8	0.0738
		OCPs-sediment	9	0.0727
Severity	Black bass	Atrazine- water	1	0.6549
		Industrial EACs- water	2	0.6278
		PAHs- water	3	0.1035
		EE2- water	4	0.0634
		PCBs- water	5	0.0548
		Hg- sediment	6	0.0507
		OCPs- sediment	7	0.0505
		OCPs- water	8	0.0504
		PAHs- sediment	9	0.0496
	Sunfish	PAHs- water	1	0.5403
		Hg- sediment	2	0.2924
		OCPs- water	3	0.1211
		PAHs- sediment	4	0.1144
		OCPs- sediment	5	0.1012
		PCBs- water	6	0.0904
		EE2- water	7	0.0767
		Industrial EACs- water	8	0.0589
		Atrazine- water	9	0.0579

Table 2
Candidate models explaining intersex occurrence (%) and severity in black bass and sunfish among sites with contaminant concentrations in water and sediment in the Yadkin-Pee Dee River of North Carolina and South Carolina. (+) indicates a positive relationship with occurrence or severity, and (−) indicates a negative relationship.

Intersex	Taxon	Model	r^2	AICc	ΔAICc	AICc Weight
Occurrence	Black bass	OCPs (Water, +); Hg (Sediment, +)	0.82	97.44	0.00	0.244
		PCBs (Water, +); OCPs (Water, +); Hg (Sediment, +)	0.92	98.22	0.78	0.165
		PCBs (Water, +); OCPs (Water, +); PAHs (Sediment, +)	0.91	98.79	1.35	0.124
		OCPs (Water, +); PAHs (Sediment, +)	0.76	100.15	2.71	0.063
		Hg (Sediment, +)	0.55	100.25	2.80	0.060
		PCBs (Water, +); Hg (Sediment, +)	0.74	100.90	3.45	0.043
		OCPs (Water, +); OCPs (Sediment, +)	0.73	101.11	3.67	0.039
		PCBs (Water, +); OCPs (Water, +); OCPs (Sediment, +)	0.89	101.13	3.69	0.039
		OCPs (Water, +); PAHs (Water, +); Hg (Sediment, +)	0.89	101.68	4.23	0.029
		OCPs (Water, +)	0.48	101.74	4.30	0.028
	Sunfish	PAHs (Water, +)	0.38	72.04	0.00	0.3452
		PCBs (Water, +)	0.16	75.15	3.11	0.0729
		Mercury (Sediment, +)	0.14	75.33	3.29	0.0667
		Industrial EACs (Water, +)	0.09	75.98	3.93	0.0483
		Atrazine (Water, +)	0.08	76.01	3.97	0.0474
Severity	Black bass	Atrazine (Water, +); Industrial EACs (Water, −)	0.69	31.12	0.00	0.4317
		Atrazine (Water, +)	0.25	34.01	2.89	0.1018
		Industrial EACs (Water, +)	0.23	34.38	3.26	0.0845
	Sunfish	PAHs (Water, +)	0.42	30.95	0.00	0.2645
		Hg (Sediment, +)	0.33	32.46	1.51	0.1243
		PAHs (Water, +); Hg (Sediment, +)	0.59	33.59	2.64	0.0707
		PAHs (Sediment, +)	0.18	34.47	3.51	0.0456
		OCPs (Sediment, +)	0.16	34.71	3.75	0.0405
		OCPs (Water, −); PAHs (Water, +)	0.53	34.87	3.91	0.0374
		PAHs (Water, +); PAHs (Sediment, +)	0.52	34.99	4.03	0.0352
		PAHs (Water, +); OCPs (Sediment, +)	0.51	35.32	4.37	0.0298
		EE2 (Water, −)	0.10	35.40	4.45	0.0286
		PCBs (Water, −); PAHs (Water, +)	0.50	35.54	4.59	0.0267

2017a; Hinck et al., 2006; Hinck et al., 2009). The overall percentage of black bass with intersex (40%) lies within the range of intersex occurrence in previous studies (Blazer et al., 2007; Blazer et al., 2014; Hinck et al., 2009; Kellock et al., 2014). Intersex was also found in sunfish, but to a lesser extent (7% compared to 40% in black bass), which is similar to findings of Lee Pow et al. (2017a). Intersex was almost nonexistent in catfish (1% of individuals at 9% of sites), which differs from findings by Barnhoorn et al. (2004), who found 20% intersex occurrence in Sharptooth Catfish (*Clarias gariepinus*) and Hinck et al. (2009), who found 7% intersex in Channel Catfish (*Ictalurus punctatus*) at 50% of sites. This variation in catfish intersex may be due to species differences or varying EAC exposures. Another study of Sharptooth Catfish (Brink et al., 2012) found subtle changes in EAC biomarkers, but no intersex condition, indicating that catfish may have limited susceptibility to the intersex condition, relative to that demonstrated in black bass.

Intersex severity also varied among sites and species. Black bass severity was, on average, higher than that of both sunfish and catfish. For example, 60% of black bass were identified as severity 3 or 4, and the majority (75%) of intersex sunfish were classified as either severity 1 or 2. One catfish (Channel Catfish) displayed the intersex condition and was identified as a severity 2. In a previous study by Blazer et al. (2007) severity in intersex smallmouth bass (*Micropterus dolomieu*), on average, ranged from 0.1 to 1.7. In 2012, Blazer et al. again found severity to be, on average, <2 in intersex smallmouth bass. These observed severities are, overall, lower than our findings in black bass in the Yadkin-Pee Dee River. Most other related studies do not include a severity index, and there is little known regarding severity among fish taxa; however, black bass may be more susceptible to intersex based on frequent higher severity rankings.

Differences in fish size, condition, and health parameters between intersex and non-intersex black bass were not detected. There were also no significant distinctions in fish parameters associated with the severity of intersex when it occurred. This lack of variation in fish size, condition, and health parameters and intersex condition is consistent with findings of other studies that found little to no significant relations (Hinck et al., 2009; Kellock et al., 2014). Hinck et al. (2009), however, found significant differences in age of fish that displayed the intersex condition, relative to non-intersex fish. We did not determine fish age or reproductive stage and, therefore, could not assess these influences in our study. Overall, the intersex condition was not related to general fish health parameters, such as length, weight, or GSI of black bass.

4.2. Endocrine active compounds

EACs were prevalent at all 11 sites examined on the Yadkin-Pee Dee River. In total, 169 contaminants were analyzed, and many of them (58%, $n = 98$) were detected in fish muscle tissue, sediment, or surface water (Table SI 2). Contaminant detection and concentration in muscle tissue of fish were generally lowest in sunfish. This may be due to the sunfishes' lower trophic level in the food web and consumption of primary consumers, compared to black bass, which are piscivorous secondary consumers and may, therefore, be exposed to more EACs through their diet (Keast, 1978; Walter III and Austin, 2003). This lower level of dietary exposure in sunfish may partially explain the observed differences in intersex occurrence between sunfish and black bass. As opportunistic omnivores with widely varying diets, it is difficult to infer a relationship between catfish diet and the intersex condition (Graham, 1999).

We detected no significant relationship between intersex condition and concentrations of muscle tissue contaminants. This lack of significance may be due to lower contaminant accumulations in the muscle tissue compared to other organs, such as the liver (Barber et al., 2006; Deb and Santra, 1997). In future studies, it may be informative to complete contaminant analysis on other organs, including the liver and gonad. Our results are comparable to those of Hinck et al. (2009), who did not find strong associations between contaminant detections in either fish tissue or environmental compartments and the intersex condition.

PAHs were detected at every site in water and sediment and associated with intersex black bass and sunfish. Sediment PAHs were the only EAC that exceeded published thresholds for aquatic life in sediment. Although levels did not exceed an ESBTU value of 1, these levels only refer to acute and chronic toxicity and may not account for reproductive impairment or reduction in overall health. PAHs are ubiquitous in the environment and reach higher levels via anthropogenic sources, such as coal- and gas-fired power plants and industrial processes (NRC, 1983). When PAHs enter aquatic environments they adsorb onto organic particulate matter, and most PAHs do so rapidly onto sediment (Tuvikene, 1995). In our study, intersex black bass were associated with higher PAH concentrations in sediment ($p = 0.003$) and water ($p = 0.0002$). PAHs adversely impact organisms through suppression of the immune system, their ability to act as anti-estrogens, and by causing alterations to reproductive function in fish (Tuvikene, 1995). Water PAHs were identified as the most explanatory variable in the sunfish intersex model and were associated with occurrence and severity in the PCA. Sediment PAHs were also important in explaining the variation in black bass intersex. The potential negative effects of PAHs on fish endocrine and immune systems may partially explain the intersex condition that we detected in the Yadkin-Pee Dee River; however, our findings are correlative and do not imply causal mechanisms, rather they may support hypothesis development and further investigation of PAH exposure and effects.

OCPs were detected at every site in the water and sediment compartments of the nine most downstream sites. However, none of the OCPs exceeded aquatic life thresholds, when they were available. Chlordanes and 4′4-DDE were the most prevalent OCPs in water and sediment. Hexachlorobenzene was detected in water and sediment at one site (Badin Lake). OCPs were used extensively in agriculture and mosquito control from the 1940s to the 1960s and are, for the most part, now banned from use in the USA. OCPs and their metabolites are extremely persistent in the environment, can bioaccumulate in organisms, and biomagnify through food webs (Skaar et al., 1981). The OCPs that we detected have been shown to bind and compete for androgen and estrogen receptors, inhibit androgen production, and block estradiol binding, thus possibly interrupting normal endocrine function (Mnif et al., 2011). Concentrations of OCPs in water and sediment were significantly higher in association with intersex black bass. Water concentration of OCPs was the highest-ranking variable explaining black bass intersex occurrence, and this may be due to bioaccumulation through integrated water-borne and dietary pathways and the black bass' position as an apex predator. Sediment OCPs were also important for black bass intersex occurrence, but were less explanatory than waterborne OCPs. Water and sediment OCPs were not very relevant in sunfish intersex modeling and PCA, possibly due to the fishes' lower trophic position.

PCBs were detected in water at downstream sites and in the sediment at five sites. All PCB levels were low and did not exceed published aquatic life thresholds. PCBs are known persistent EACs and cause endocrine disruption and alter reproductive capabilities in fish (Baldigo et al., 2006; Hillis et al., 2015; Simmons et al., 2014). Elevated PCB concentrations in sediment and water were associated with black bass intersex, and PCB concentrations in black bass muscle tissue explained intersex severity. Waterborne PCBs were equally important in explaining black bass and sunfish intersex occurrence, and this is presumably due to their endocrine disruption capabilities, persistence, and ability to bioaccumulate into all trophic levels of a food web.

CUPs were detected at 10 of 11 sites in water and at 4 sites in sediment. Atrazine, simazine, and metolachlor were frequently detected in the water, and atrazine was the only CUP detected in sediment. CUPs are applied on agricultural lands, and agriculture accounts for 24% of land use in the Yadkin-Pee Dee River basin (Homer et al., 2015). CUPs are capable of endocrine disruption by acting as anti-androgens, strong estrogens, and receptor activators (Mnif et al., 2011). With existing

pesticide use, and probable future use in North Carolina and South Carolina, CUPs will most likely persist in the aquatic environment for the foreseeable future. The toxicity of many CUPs has not been fully investigated for aquatic organisms, and even less is known about the effects of pesticide mixtures or interactions with other EACs. In our PCA, CUPs tracked closely with industrial EACs, but were not explicitly related to intersex occurrence or severity in black bass or sunfish. Atrazine (the representative CUP for modeling) was not identified as particularly explanatory for intersex occurrence in black bass or sunfish, and its concentration was not significantly different between intersex and non-intersex black bass. This may be because of the low concentrations and overall detections of atrazine or CUPs in general. However, an important limitation was that water (PSD) sampling occurred for a one-month duration and CUP application times may vary throughout the year. A more comprehensive CUP field assessment coupled with laboratory assessment during critical windows of fish development may further elucidate CUP effects on fish health in natural environments (Guillette et al., 1995; Lee Pow et al., 2017b). Atrazine was a predominant variable in black bass intersex severity, and this may indicate an influential role on EAC effects in areas where initial disruptions occur (areas of intersex occurrence).

Industrial EACs and estrogens have been increasingly investigated because of their potential to cause endocrine disruption at low concentrations (ng/g, ng/L; Mills and Chichester, 2005). In our study system, industrial EACs (BPA and nonylphenol), E2β-Eq, and EE2 were detected at most sites and were often found in higher concentrations downstream of, or in association with, urbanized areas. Six other hormones were not detected in the river, but this may be due to instrument and method detection limits. In the future, improved methods for detecting estrogens and other EACs in environmental samples will be critical, because EACs are suggested to be capable of disruption at very low concentrations or within mixtures (Brian et al., 2007; Kolpin et al., 2002). EE2 is a synthetic estrogen and is over 10-fold more potent than estradiol for several estrogen receptors and has a low PNEC threshold (0.1 ng/L), making its presence in the river of great concern (Caldwell et al., 2012; Thorpe et al., 2003). Industrial EACs and estrogens were not important explanatory variables in PCA or modeling of black bass or sunfish intersex, but this may be due to the lack of detections and our lab constraints, and they remain relevant aquatic contaminants to be considered in future studies. In addition, similar to CUPs, these compounds may temporally fluctuate in the environment with river flow and input, and long-term monitoring may be required to determine concentration trends.

Within fish muscle tissue and sediment samples, up to 18 different metals were detected at nearly all sites. Most metal concentrations did not exceed effect thresholds; however, Mn and Cd exceeded thresholds for aquatic life in sediment, and Hg in fish tissue exceeded human health consumption thresholds in several specimens. Even at low concentrations, Cd enters the food web and bioaccumulates, and it can interfere with steroid hormones of both male and female reproductive organs by disrupting biosynthesis of hormones and by binding to both androgen and estrogen receptors (Georgescu et al., 2011). Mn is a naturally occurring metal but can be found at higher levels in the environment because of anthropogenic influences such as industrial waste discharges, combustion of fossil fuels, mining, and leaching from landfills (ATSDR, 2012). Mn has been studied very little in fish; however, a study on rats found that Mn altered the production and secretion of reproductive hormones (Pine et al., 2005). Hg and associated methylmercury can cause adverse reproductive effects through suppressed and altered hormone levels (Tan et al., 2009). Sediment Hg (the representative metal chosen for PCA and modeling) tracked with intersex severity in sunfish and, to a lesser extent, intersex occurrence in black bass when examining PCA results. Modeling identified Hg as high importance in black bass and sunfish intersex occurrence and sunfish intersex severity, and other metals in black bass muscle tissue explained the occurrence (Cu) and severity (Al, Mn, Se) of intersex in individual fish. Due to their explanatory power in modeling, Hg and other endocrine disrupting metals are potentially influential in causing the intersex condition.

4.3. Riverine contaminants and intersex trends

No distinct increasing upstream to downstream longitudinal trend in the fish intersex condition was observed within the Yadkin-Pee Dee River. Rather, site-related patterns were observed, which may be due to variable inputs of EACs, fluctuations in habitat characteristics and fish assemblages, ability to capture individuals, and the dynamic nature of the river system and the presence of multiple large reservoirs. Some waterborne EACs (e.g., PCBs, nonylphenol, EE2, and BPA) had elevated concentrations at downstream sites, and these may be due to influx from smaller tributary streams, which may be influenced by wastewater effluent from variable-sized municipalities. Sediment EACs did not exhibit any longitudinal trend, and this may be due to sediment accumulation occurring locally from pollution sources, the possible lack of EAC degradation in sediment, or retention of contaminated sediment in the numerous mainstem river reservoirs. Fish tissue contaminant concentrations also did not exhibit longitudinal trends, again presumably because of varying species and EAC inputs along the river. Contaminants were often found to co-occur within organisms and this co-occurrence, and possible additive effects, may warrant future study. Hg concentrations in fish muscle tissue were elevated at the most downstream sites, which is likely due to the stimulation of methylmercury production in the sediments of low pH areas (i.e., swamps) that drain into the lowland reaches of the river (Jernelov, 1972).

Fishes may function as sentinels for human populations and may be used to investigate human health. EACs, and particularly estrogenic EACs, have been found in sediment, surface water, ground water, wastewater, aquatic life, and even the atmosphere (Campbell et al., 2006). Reducing contaminant influx would protect both human health and ecosystems, and source identification and best management practice development are important first steps. Improved wastewater technologies, reduced outputs from industrial and power production facilities, low impact agriculture practices, and public awareness of EAC effects on the environment may be beneficial first steps.

4.4. Future implications

Long-term exposure to EACs, even at low concentrations, has raised concern for population-level effects of fishes (Nash et al., 2004). Although population-level threats were not examined in this study, EACs have been shown to cause population collapse of Fathead Minnow (*Pimephales promelas*) in a lake environment (Kidd et al., 2007). Anecdotally, we found black bass species, the taxon with the highest intersex prevalence, to be rare in the river during field sampling. Monitoring of intersex condition, overall fish health and population abundance, and EAC concentrations in the Yadkin-Pee Dee River will be required to detect reproductive failure or other population-level impacts. With probable increased anthropogenic impacts and new chemical contaminants being produced and released into the environment, it will be vital to investigate EACs in the environment and their subsequent influences on fish health and populations. In addition to the EACs examined in this study, personal care products, surfactants, and other potent emerging contaminants may be influential in endocrine disruption, fish health, and population viability. Methods to reduce organisms' exposure to these contaminants would be beneficial; including improved wastewater treatment technologies, reduction in runoff, and decrease of overall chemical use, which would reduce inputs of EACs into the aquatic environment. For example, Hicks et al. (2017), found that upgrading wastewater treatment, using a nitrifying activated sludge treatment process, resulted in major reductions in the intersex condition and concentrations of contaminants in effluent.

Within this large, regulated, Atlantic slope river system, the intersex condition of fish, especially black bass and sunfish, appears to be correlated with both sediment and waterborne EACs. There is a possibility that mixtures of EACs, working in an additive or synergistic manner, may increase their toxicity to organisms (Rajapakse et al., 2002). This mixture of EACs was also evident in a study by Lee Pow et al. (2017a), in which it appeared that different EACs influenced fish intersex condition and its severity. Further, the absence of intersex does not necessarily indicate that a fish is reproductively viable and thus, monitoring other reproductive biomarkers and reproductive success may be considered when evaluating fish population health. Applying quantitative methods, we identified which variables are most plausible for explaining intersex occurrence in black bass and sunfish. This approach serves as a valuable initial exploratory exercise for identifying relationships between EACs and the intersex condition, and further research may infer causal mechanisms. Our findings document a strong correlation between EACs and intersex and provide the basis for future research to examine molecular mechanisms involved with the development of the intersex condition. Such mechanistic research, along with additional field research, may further elucidate individual- and population-level effects of contaminants in aquatic systems, leading to sustainability of wild fish populations and may identify best management practices.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.06.071.

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