Environmental Toxicology and Chemistry—Volume 40, Number 8—pp. 2206–2219, 2021
Received: 25 November 2020 | Revised: 23 December 2020 | Accepted: 7 April 2021

Environmental Toxicology

Survival and Contaminants in Imperiled and Common Riverine Fishes Assessed with an In Situ Bioassay Approach

Casey A. Grieshaber,[a,*] W. Gregory Cope,[b] Thomas J. Kwak,[c] Tiffany N. Penland,[a] Ryan J. Heise,[d] and J. Mac Law[e]

[a]North Carolina Cooperative Fish and Wildlife Research Unit, Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina, USA
[b]Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina, USA
[c]US Geological Survey, North Carolina Cooperative Fish and Wildlife Research Unit, Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina, USA
[d]Duke Energy, Environmental Services, Huntersville, North Carolina, USA
[e]Department of Population Health and Pathobiology, North Carolina State University, Raleigh, North Carolina, USA

Abstract: An in situ bioassay approach was used to determine whether aquatic contaminant stressors in a large Atlantic river ecosystem affect the survival of 3 fish species: the largemouth bass (*Micropterus salmoides*, juveniles), the fathead minnow (*Pimephales promelas*, adults), and the robust redhorse (*Moxostoma robustum*, juveniles). Hatchery-propagated fish were placed into cages to assess site-specific survival in the Yadkin-Pee Dee River of North Carolina and South Carolina, USA. Contaminants were measured in caged fish and sediment and surface water at each site. No apparent longitudinal trends in fish survival were detected, and contaminant concentrations varied among sites. Juvenile largemouth bass and robust redhorse did not survive past 13 and 23 d, with corresponding Kaplan-Meier median survival estimates of 9.7 and 12.1 d, respectively. Survival of adult fathead minnows deployed in cages alongside the juvenile fish averaged 43% at the end of the 28-d exposure, with a 22-d median survival estimate. The intersex condition, an indicator of endocrine disruption, was not observed in any adult fathead minnow. Contaminant accumulation in surviving fathead minnows was apparent, with highest accumulated concentrations of polychlorinated biphenyls (34.6–93.4 ng/g dry wt), organochlorine pesticides (19.9–66.1 ng/g dry wt), and mercury (0.17–0.63 µg/g dry wt). Contaminants and other water quality stressors in this river system appear to detrimentally impact juvenile fish survival, with presumed effects at the fish assemblage and community levels. *Environ Toxicol Chem* 2021;40:2206–2219. © 2021 SETAC

Keywords: Polychlorinated biphenyls; Mercury; Kaplan-Meier; Endocrine disruption; Robust redhorse

INTRODUCTION

Aquatic ecosystems are vulnerable to anthropogenic impacts because they receive and sequester many contaminants (Scholz and Mayer 2008). With increasing human populations and associated impacts, understanding and protecting aquatic ecosystems are critical. Contaminants are released from industrial, agricultural, and municipal activities; and the magnitude and frequency of releases are likely to grow as the human population increases (Daughton and Ternes 1999). Many diverse assemblages of aquatic animals are at risk, and anthropogenic threats continue to increase (Warren et al. 2000). Contaminants that enter aquatic ecosystems can be detrimental to the health and survival of organisms. Those that are commonly found in aquatic systems include polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs), pharmaceuticals, bisphenol A (BPA), and heavy metals (Muthumbi et al. 2003; Hinck et al. 2009).

Contaminants can cause endocrine disruption, leading to health problems such as decreased reproductive success, increased levels of stress, and reduced ability to combat stress, altered behaviors, and death (Kidd et al. 2007; Colman et al. 2009; Tan et al. 2009). Exposure to contaminants during critical periods of development can also be important in determining how an organism will respond (Barton and Andersen 1998; Lee Pow et al. 2017b). In addition to causing impaired health and mortality, contaminants, especially those with estrogenic properties, can cause the occurrence of the intersex condition (Bahamonde et al. 2013). Intersex, as defined in the present study, is the presence of female germ cells within a

This article includes online-only Supplemental Data.
* Address correspondence to casey.grieshaber@gmail.com
Published online 6 May 2021 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.5104

predominantly male gonad (Nolan et al. 2001). Research on fish intersex has previously been conducted globally and specifically in the United States by Hinck et al. (2009). Their survey examined intersex in fishes at 111 sites across the United States and included 3 sites on the Yadkin-Pee Dee River of North Carolina and South Carolina. Hinck et al. (2009) found that fish intersex occurrence in male largemouth bass ranged from 8 to 91% at sites across the country and was highest in the Yadkin-Pee Dee River (91%, Pee Dee River at Bucksport, SC, USA). That study, along with the knowledge of impacts caused by dense human populations, industry, wastewater treatment, agriculture, and concentrated animal feeding operations in this river basin, motivated our study to examine contaminant impacts on the survival and health of riverine fishes. Fish are commonly considered an indicator of aquatic ecosystem health because they are continuously exposed to the contaminants in their environments (Kwak and Freeman 2010; Blazer et al. 2012). By examining fish health and survival, researchers can assess water quality stressors and the overall condition of the river ecosystem.

The Yadkin-Pee Dee River is an important study system because it supports a population of an imperiled fish species, the robust redhorse (*Moxostoma robustum*). Robust redhorse is a long-lived sucker species that inhabits only 3 drainages in the southern United States. In the Yadkin-Pee Dee River, the species is impacted by habitat alteration (Fisk et al. 2015), and recent estimates of this population are 96 reproducing adults (95% confidence interval 75–116 [North Carolina Wildlife Resources Commission 2019]). Although the river is altered, research suggests that adequate physical habitat (i.e., water depth and velocity, substrate, and cover) for this species exists in the Yadkin-Pee Dee River (Fisk et al. 2015), indicating that water quality may be a greater stressor than physical habitat availability.

To examine the effects of contaminants on fish in a field setting, we designed the present study employing in situ bioassays, which allow for ecologically relevant toxicity testing, in a time- and cost-effective manner. Hewitt et al. (2006) and Cope et al. (2011), as well as other investigators, have used this exposure method with fish species in river ecosystems; and we adopted similar procedures. These bioassay exposures allow juvenile and adult captively propagated fish to be exposed to the river environment in a controlled manner, and provides investigators the ability to monitor survival and determine contaminant accumulation at the end of the exposure. This provides researchers an understanding of what environmentally relevant conditions exist, their integrated impacts on organism health and survival, and overall water and sediment quality (Goulden 1999). Research has examined intersex and contaminant effects in adult wild fish (e.g., black bass, sunfish, and catfish); however, less has been done to examine contaminant effects on juvenile fish or on fish through in situ bioassays (i.e., controlled exposures of organisms to ambient field conditions). Further, juvenile fish life stages are known to determine recruitment and ultimately population abundance through a survival bottleneck, which is especially significant for imperiled species (Schiemer et al. 2003; Humphries et al. 2013).

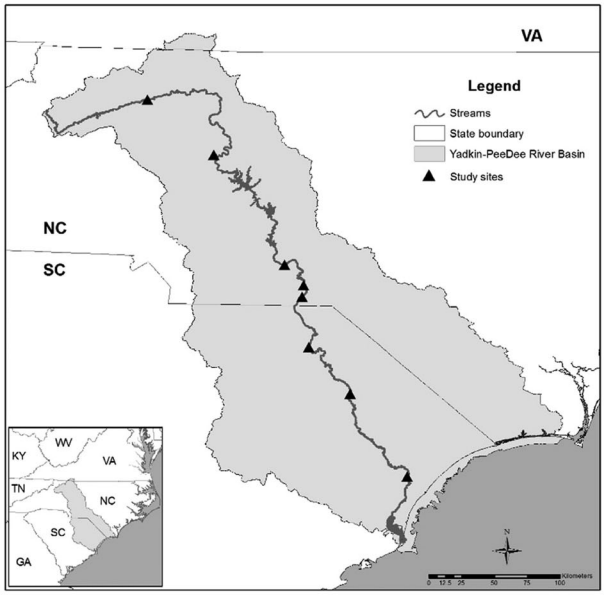
The present study utilized in situ bioassays to 1) examine survival of 3 fish species in the river ecosystem, 2) determine whether the intersex condition or other obvious health abnormalities develop in exposed fish, 3) assess water and sediment quality, and 4) determine whether contaminant accumulation occurs in river-exposed fish over a relatively short (28 d) duration.

METHODS

Site selection and test design

Eight riverine sites located along the Yadkin-Pee Dee River in North Carolina (5 sites) and South Carolina (3 sites), USA, were selected for in situ bioassays (Figure 1). Sites exhibited varying levels of anthropogenic influence, land use, and habitat types (Sackett et al. 2015). They were selected to represent the longitudinal river continuum and for availability of boat access and adequate depth and flow to maintain suitable dissolved oxygen and food items to flow through the fish enclosure. Three of the sites were previously sampled by Hinck et al. (2009) for the intersex condition and associated contaminants in fish (Hinck et al.: PRB 336, PRB 337, PRB 338; present study: 74 Bridge, Pee Dee, Bucksport).

The assays included the use of 3 fish species: fathead minnow (*Pimephales promelas*), largemouth bass (*Micropterus salmoides*), and robust redhorse. Fathead minnow are commonly used in toxicological studies, are known to be tolerant to environmental conditions and contaminants, and have been used in similar bioassays (Dwyer et al. 2005; Cope et al. 2011). Largemouth bass are a common sportfish species in the Yadkin-Pee Dee River, and robust redhorse are listed as state endangered in North Carolina and Georgia and require additional research to determine population stressors (Grieshaber et al. 2018; Robust Redhorse Conservation Committee 2018; Penland et al. 2020). Two separate and sequential in situ bioassays were conducted for 28-d durations. The first bioassay utilized adult fathead minnow and juvenile largemouth bass, and the second included adult fathead minnow and juvenile robust redhorse. Because of the young age and immaturity of the largemouth bass and robust redhorse, sex determination was not possible prior to the present study. Although all adult fathead minnow used for the present study were intended to be males, later histology determined that the fish used were both males and females. At each of the 8 sites, 3 cages per species were deployed, containing 20 fish each. Survival and physicochemical properties of river water were monitored every 3 d for the duration of the 28-d bioassay. During each bioassay, in addition to riverine bioassay cages, 3 cages of juvenile largemouth bass and 3 cages of juvenile robust redhorse were placed in small hatchery ponds at the North Carolina Wildlife Resources Commission Watha State Fish Hatchery and the McKinney Lake State Fish Hatchery, respectively. These reference treatments allowed us to determine caged fish survival in hatchery ponds compared to that at riverine sites. All fish used in the present study were handled, held, and processed according to North Carolina State



Site	Latitude	Longitude	State
Ronda	36.21601	-80.937164	NC
Rt. 801	35.838287	-80.484752	NC
Red Hill	35.0847222	-79.9986111	NC
74 Bridge	34.9455556	-79.8691667	NC
Diggs Tract	34.8652778	-79.8791667	NC
Society Hill	34.524154	-79.832815	SC
Pee Dee	34.203845	-79.547676	SC
Bucksport	33.661734	-79.153058	SC

FIGURE 1: Map of study sites located on the Yadkin-Pee Dee River, North Carolina and South Carolina, for in situ bioassays with fathead minnow (*Pimephales promelas*), largemouth bass (*Micropterus salmoides*), and robust redhorse (*Moxostoma robustum*), June through August 2014.

University Institutional Animal Care and Use Committee protocol number 14-064-O.

Fish transport and deployment

Fathead minnow used in the bioassays were purchased from Aquatic Biosystems, were approximately 8 to 10 mo old, and were housed in their "clean systems" with minimal potential exposure to contaminants. Based on a subsample of baseline individuals, which were anesthetized at the initiation of the bioassay, mean ± standard error (SE) fathead minnow total lengths were 65.44 ± 0.77 mm for the first assay and 63.60 ± 1.10 mm for the second assay. Fathead minnow weights were 3.16 ± 0.14 g for the first assay and 2.91 ± 0.15 g for the second assay. Largemouth bass were obtained from the Watha State Fish Hatchery and were approximately 30 d old at the beginning of testing. Largemouth bass were 27.9 ± 0.34 mm long (total length, mean ± SE) and weighed 0.199 ± 0.013 g. Robust redhorse were acquired from the McKinney Lake State Fish Hatchery and were the captively propagated offspring of adults collected from the Yadkin-Pee Dee River. Robust redhorse were approximately 45 d old at the beginning of testing and were 38.60 ± 0.26 mm long (total length, mean ± SE) and weighed 0.519 ± 0.011 g.

At the initiation of each bioassay, fish were obtained in-person from the respective state hatchery or shipped via overnight courier from Aquatic Biosystems and placed into a fish transport box equipped with a compressed oxygen tank that provided aeration with adequate temperature acclimation. Water quality was routinely monitored for dissolved oxygen (milligrams per liter) and temperature (Celsius) in the fish transport box, and mortality was monitored throughout transportation during deployment to bioassay cages. Fish were transported to each riverine site where 60 of each species (60 largemouth bass and 60 fathead minnow for the first bioassay and 60 robust redhorse and 60 fathead minnow for the second bioassay) were removed from the fish transport box and placed into 19-L buckets for a brief (1 h) acclimation period. Over the 1-h acclimation period, river water (~1 L) was added to the buckets every 15 min. Twenty fish (of the same species) were placed into each cage with 3 replicate cages per species per site. All fathead minnows were purchased as presumptive males, but sex was not confirmed on fish samples prior to stocking in cages because of added holding time and handling stress, as well as other logistical constraints. Fish density in cages ranged from approximately 0.9 to 13.6 g/L, depending on the species within each cage. These loading densities are similar to those recommended for laboratory flow-through toxicity tests (ASTM International 2007). Cages were held in buckets with river water, transported by boat to the riverine locations, and deployed. Bioassays were conducted during June and July 2014.

Cages were similar to those used by Hewitt et al. (2006) and Cope et al. (2011) and constructed of a plexiglass tube (length, 25 cm; outer diameter, 15 cm) with 1.6-mm holes drilled into the sides to ensure flow. Nitex screen (1-mm mesh) was secured to each end of the cage with stainless steel circular clamps. Cages were fastened to a concrete block using cable, secured to solid structure on the riverbank with parachute cord, and deployed approximately 25 cm off the river bottom. Each replicate cage was uniquely identified by unique combinations of colors and numbers of zip ties. Cages were placed nearly parallel to the riverbank to reduce drag and allow water flow through them, thus keeping conditions inside the cages as comparable to outside river conditions as possible.

Additional fish, which served as the baseline subsample, were returned to North Carolina State University, where they were euthanized using a lethal overdose of pH-buffered tricaine methanesulfonate (MS-222). Each fish was weighed (grams) and measured (total length, millimeters); and for

fathead minnows, the presence or absence of nuptial tubercles was recorded. A subsample of fish from this group was then preserved in modified Davidson's fixative (35.15% distilled water, 31.35% ethanol, 22% formalin [37–40%], and 11.5% glacial acidic acid) for 24 h before being transferred to 70% ethanol for histological examination. Remaining fish were randomly allocated into groups of approximately 25 each and stored frozen at –20 °C for contaminant analysis.

Bioassay monitoring and termination

Approximately every 3 d throughout the in situ bioassays, cages were monitored for fish mortality, biofouling, and sediment accumulation. Each cage was carefully pulled to the surface, and the cage was assessed and cleaned, as necessary. If a large mortality event had occurred, dead fish were removed from the cage. Any vandalism or abnormal conditions were noted. During each monitoring event, water physicochemical variables, including water temperature, conductivity, salinity, dissolved oxygen, and pH, were measured as close to the cages as possible using a YSI Model 556 multiprobe instrument and a Beckman Coulter Phi 400 series, model 410 pH, meter. Although current velocity and river discharge were not monitored during the bioassays, US Geological Survey gaging stations along the river indicated that flows were within average ranges and that the river was not in drought or flood stage at any point during our study.

If complete mortality in a replicate occurred before the end of the 28-d bioassay, the cage was removed from the river. At the end of the 28-d duration, any remaining cages were retrieved, the total number of live fish was recorded, and cages with live fish were labeled and transported to North Carolina State University using an oxygenated fish transport box. Any additional mortality during transportation was noted. Remaining fish were euthanized with buffered MS-222, and subsamples of fish from each site (3–10 depending on survival) were preserved in modified Davidson's fixative and later transferred to an ethanol solution. All other fish were frozen at –20 °C for contaminant analysis. Presence or absence of nuptial tubercles was noted for fathead minnows.

Fish histopathology

Preserved fish were transported to the North Carolina State University College of Veterinary Medicine Histopathology Laboratory for sectioning and microscopic evaluation. Whole fish (some with head or tail excluded depending on size) were embedded in paraffin wax, sectioned at 5 µm with a microtome, and stained with hematoxylin and eosin. Following histological preparation, a certified fish pathologist examined the slides with light microscopy for the occurrence and severity of intersex and presence of food in the gut. Any abnormalities, such as signs of infection and lesions, were noted if found.

Contaminant analyses

Contaminants were measured in the water and sediment of the Yadkin-Pee Dee River at each of the 8 sites. Surface water was analyzed from grab samples (2-L bottles) and passive sampling devices (PSDs), and sediment was analyzed from grab samples of bottom sediments, as described by Grieshaber et al. (2018) and Penland et al. (2018). Fathead minnow whole-body composite samples were analyzed for contaminants in both baseline and river-exposed individuals. Largemouth bass and robust redhorse did not have enough fish survive to the end of the bioassay to be examined for tissue contaminants.

To determine estrogenicity of the water, grab water samples were collected in solvent-rinsed and baked 2-L amber glass bottles during the fish bioassay period. At each site, a subsurface sample was collected and acidified to a pH of 2 to prevent bacterial degradation. Samples were placed on ice and transported to North Carolina State University, 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 assay, which uses human breast adenocarcinoma cells and 17β-estradiol (E2β) standard to determine E2β equivalent concentrations (E2β Eq, nanograms per liter). The T47D-Kbluc assay measures total estrogenic activity but does not differentiate among or identify specific estrogens of varying potencies. Full descriptions of the assay and extraction process are detailed by Yost et al. (2014).

At each site, PSDs were deployed for approximately 28 d to determine time-weighted estimates of waterborne contaminant concentrations. Two types of PSDs were deployed. Low-density polyethylene strips were utilized to sample OCPs, PCBs, and PAHs. A sorbent-containing cartridge, universal PSD, was used to sample current-use pesticides (CUPs), hormones, and industrial contaminants (Supplemental Data, Table SI1). A coarse-mesh plastic container housing both types of PSDs was connected to the riverbank at the bioassay site. Each PSD container was suspended in the water column by a buoy and kept stationary with an attached brick. After approximately 28 d, PSDs were collected from each site, removed from their container, wrapped in baked aluminum foil, sealed in clean plastic bags, placed on ice, transported to North Carolina State University, and stored at –20 °C until extraction. Complete details of PSD protocols and extraction procedures can be found in the Supplemental Data and Lee Pow et al. (2017a). The PSDs were deployed once during the bioassay period, from mid-June to mid-July.

Sediment samples were collected at each site within 2 mo of the bioassays. Two composite samples (3–5 grabs each) of surficial (top 5 cm) sediment were collected near the riverbank using a stainless steel scoop and tray and stored in 250-mL amber glass jars. Sediment was transported on ice and stored at –20 °C until extraction. RTI International analyzed samples for 22 metals (Supplemental Data, Table SI1). Mercury (Hg) concentration was determined using a Milestone DMA-80 direct Hg analyzer. Other metals were evaluated with a modified version of Method 3050B (US Environmental Protection Agency 1996) and a Thermo X-Series II inductively coupled plasma-mass spectrometer (ICP-MS) or a Thermo iCAP6500 ICP-atomic emission spectrometer (OES) depending on the concentration

of the analyte present in the sample. The Analytical Toxicology Laboratory at North Carolina State University determined the concentrations of CUPs, OCPs, PAHs, and PCBs (Supplemental Data, Table SI1). Sediment samples were extracted with dichloromethane by means of pressurized fluid extraction using a Buchi Speed Extractor E-916. Extracts were cleaned using gel permeation chromatography (GPC). Before chemical analysis, extracts were concentrated to 0.5 mL under a gentle stream of nitrogen.

Whole-fish samples were homogenized and stored frozen at –80 °C until further processing. One fathead minnow composite sample was prepared from each site at the conclusion of each bioassay, as well as 3 pretreatment baseline samples for each bioassay. No samples of largemouth bass or robust redhorse were analyzed for contaminants because of insufficient survival. In preparation for contaminant analyses, samples were lyophilized and manually homogenized. RTI International analyzed fish tissue samples for 22 metals (Supplemental Data, Table SI1). The Hg concentration in tissue samples was determined using a Milestone DMA-80 direct Hg analyzer. Other metals were analyzed with a modified version of Method 3050B (US Environmental Protection Agency 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 North Carolina State University analyzed samples for PCBs and OCPs (Supplemental Data, Table SI1). Lyophilized muscle tissue was extracted with dichloromethane by means of pressurized solvent extraction using a Buchi Speed Extractor E-916. Extracts were then cleaned using GPC and processed through Florisil solid-phase extraction cartridges. Extracts from PSDs and sediment samples were analyzed for 42 PAHs, 28 OCPs, 21 PCBs, and 47 CUPs. The PSDs were also used to determine concentrations of 7 estrogen hormones and 2 industrial contaminants. Whole-fish composite sample extracts were analyzed for 21 PCBs and 28 OCPs (Supplemental Data, Table SI1). Current-use pesticides, 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 contaminants 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 expressed as nanograms per liter, and sediment and whole-fish contaminant results are expressed as nanograms per gram dry weight for organics and micrograms per gram dry weight for metals. Detection limits 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 (based on an equivalent 30-d PSD deployment). Detection limits for sediment and whole-fish contaminants were 0.1 ng/g for OCPs and PCBs, 1.0 ng/g for PAHs, and 2.0 ng/g for CUPs (all dry wt).

Chemicals were evaluated both as individual analytes and as total concentrations of classes of chemicals, when appropriate. For PAHs, individual analytes were examined, and a total sum of analytes was calculated to compare to predicted-effect concentrations (PECs). In water and sediment samples, PAHs were also analyzed using the equilibrium partitioning sediment benchmark toxicity units (ESBTU) method because it incorporates the additive nature of PAH toxicity and the bioavailability of PAHs due to organic carbon in the sediment (US Environmental Protection Agency 2012). Of the 42 PAHs analyzed, 34 have PAH potency divisors published by the US Environmental Protection Agency (2012). Using the potency divisor, the determined concentration, and (for the sediment sample) the carbon content of the sediment sample, a toxic unit was determined for each PAH (acute and chronic values). Then, all 34 PAH toxic units were summed to an overall PAH toxic unit value; a value <1.0 indicates that it is unlikely for the PAHs to cause adverse effects to aquatic life, and a value >1.0 indicates that aquatic life may be negatively affected.

Statistical analysis

Statistical analyses were completed using JMP Pro 14.2 and SAS 9.4 (SAS Institute), with a statistical significance level (α) of 0.05. Fish survival was modeled using the Kaplan-Meier (K-M) product limit estimator (Proc Lifetest) to estimate median survival during the 28-d bioassay (Kaplan and Meier 1958). Differences in survival among test sites were tested with the log-rank test. Correlation analysis was used to assess the strength of relationships among environmental conditions, contaminant concentrations, and the survival of fathead minnow using Spearman's rank-order correlation (r_s). Nonparametric correlation was employed because the majority of data did not conform to a normal distribution (Shapiro-Wilk test) and sample sizes were low. Tukey's multiple comparison tests and analyses of variances were performed to determine significant differences in length and weight of fish between baseline and fish deployed at test sites that survived to the conclusion of the bioassay exposures.

RESULTS

Fish survival and growth

Mean survival among all river sites during the first bioassay was 58% for fathead minnow and 0% for largemouth bass. Fathead minnow survival remained high (>90%) for the first 20 d of the bioassay at most sites. Ronda, the most upstream site, exhibited the lowest survival with only 15% of fathead minnows surviving to day 28. Fathead minnow survival was >50% for all other sites (Figure 2). Fathead minnow K-M survival functions were significantly different among sites ($\chi^2 = 30.65$, $df = 7$, $p < 0.0001$). Almost no largemouth bass survived past day 9 at most sites. By day 16, no surviving largemouth bass were present. Median survival duration was 9.7 d for largemouth bass and 24.9 d for fathead minnow (Figure 3). Largemouth bass 28-d survival at the hatchery

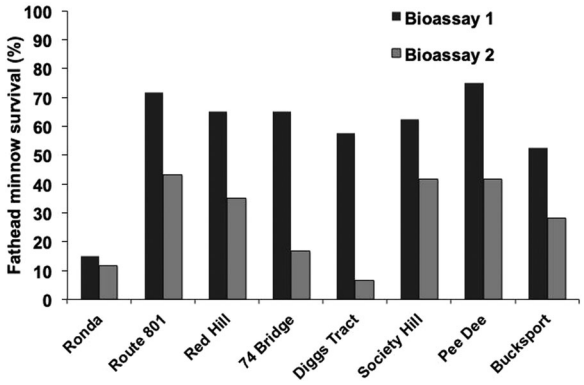


FIGURE 2: Fathead minnow (*Pimephales promelas*) survival at each riverine site, at day 28 of in situ bioassays 1 and 2.

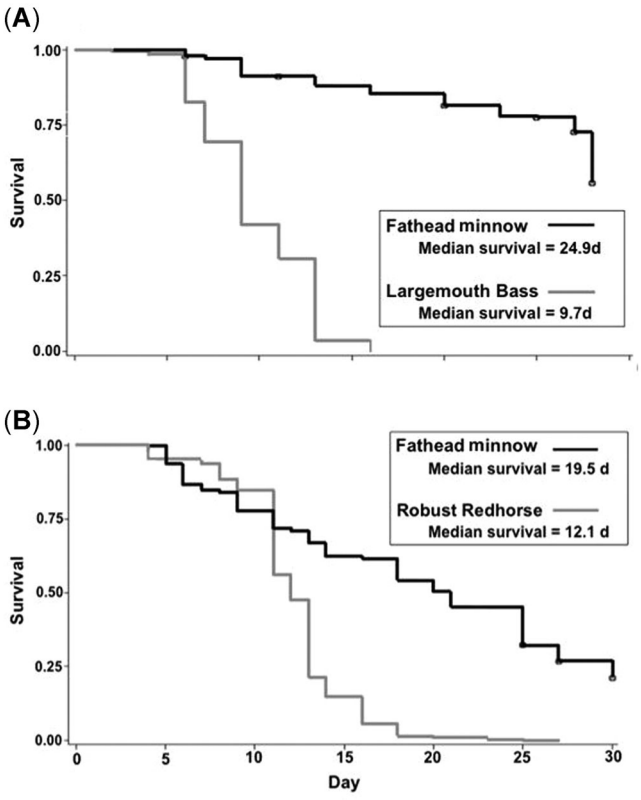


FIGURE 3: Kaplan-Meier survival distribution function for fathead minnow (*Pimephales promelas*), largemouth bass (*Micropterus salmoides*), and robust redhorse (*Moxostoma robustum*) at all river sites during in situ bioassays 1 (**A**) and 2 (**B**).

reference site was 70%. Largemouth bass K-M survival functions were significantly different among river and hatchery reference sites ($\chi^2 = 148.18$, $df = 8$, $p < 0.0001$).

Fathead minnow survival trends during the second bioassay were generally similar to those of the first, except for higher mortality at the beginning of the bioassay and overall lower survival in the second bioassay. Mean 28-d survival in the second bioassay was 28.1% for fathead minnow and 0% for robust redhorse. By day 12, fathead minnow at 4 of the sites had experienced ≥50% mortality. Survival of fathead minnow was lowest at the Ronda, 74 Bridge, and Digg's Tract sites (Figure 2). Fathead minnow K-M survival functions were significantly different among river sites ($\chi^2 = 140.38$, $df = 7$, $p < 0.0001$). Robust redhorse in the river did not survive to the end of the 28-d test; their survival was >60% before day 9, but following day 9, survival at most sites declined precipitously. Median survival duration was 12.1 d for robust redhorse and 19.5 d for fathead minnow (Figure 3). Survival of robust redhorse (28 d) at the hatchery reference site was 67%. Robust redhorse K-M survival functions were significantly different among river and hatchery reference sites ($\chi^2 = 139.86$, $df = 8$, $p < 0.0001$).

Fathead minnows that survived the 28-d bioassay provided insightful comparisons to baseline fish measurements. In the first bioassay, river-exposed fish length was significantly lower at 2 sites, and weight was significantly lower at all sites, relative to baseline measurements (Figure 4). In the second bioassay, there was a significant difference in lengths of fish at only one river site and baseline fish, but exposed fish weighed significantly less than baseline fish (Figure 4).

Fish intersex and histopathology

The intersex condition was not detected in any fathead minnow, largemouth bass, or robust redhorse examined. From the first bioassay, a total of 12 baseline fathead minnow individuals were assessed for the intersex condition. Another 76 fathead minnows (5–15 from each river site) were assessed for intersex, post–river exposure. Intersex was not detected in any fathead minnows. Baseline largemouth bass ($n = 7$) and hatchery site–exposed largemouth bass ($n = 5$) were also examined for the intersex condition, and it was not found. From the second bioassay, a total of 11 baseline fathead minnows were assessed for the intersex condition. Another 60 fathead minnows (2–16 from 7 river sites; low survival of fathead minnows at the Digg's Tract site) were assessed for intersex, post–river exposure. Intersex was not detected in any fathead minnows. Baseline robust redhorse ($n = 5$) and hatchery site robust redhorse ($n = 5$) were also examined, and no intersex was observed.

Using light microscopy, fish gastrointestinal (GI) tracts were examined for the presence of food items to determine if starvation was a potential factor in the observed mortality. The GI tracts of fathead minnows from all riverine sites and of robust redhorse and largemouth bass taken from the hatchery sites were filled with organic material, indicating that feeding was occurring. Mineralization deposits were visually observed in the testes of fathead minnow, although these deposits were not considered to represent any adverse health condition and were present in both baseline and river-exposed fish. There was no discernible evidence of confounding disease or infection (lesions, parasites, etc.) in river-exposed fathead minnows.

All baseline fathead minnow had noticeable to large tubercles, and some had enlarged dorsal epithelial fat pads (all male characteristics). On termination of the bioassay, all river-exposed fathead minnows had no tubercles or had very small, light tubercles and light or nonexistent dorsal epithelial fat pads.

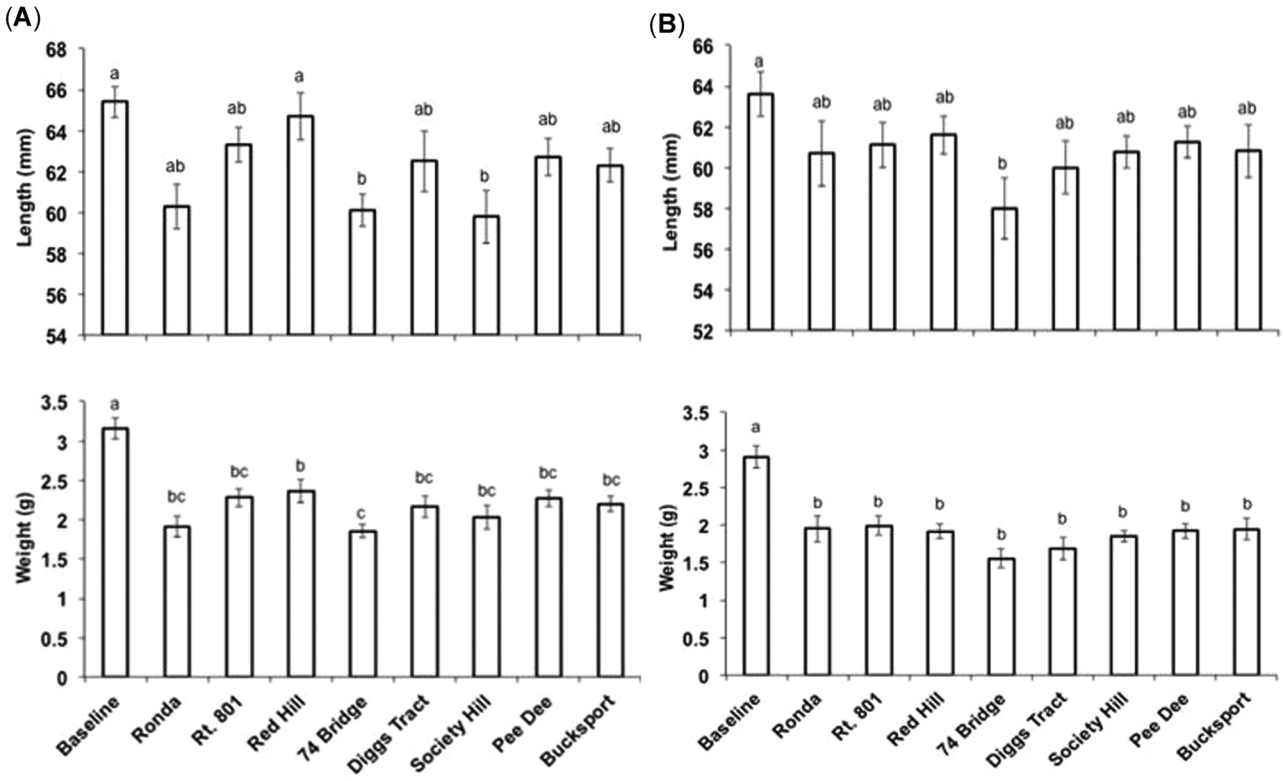


FIGURE 4: Mean (±standard error) fish total length (above) and weight (below) of baseline fathead minnow (*Pimephales promelas*) and those recovered from cages at each site for bioassay 1 (**A**) and bioassay 2 (**B**). Sites with the same letter indicate no significant difference ($p > 0.05$).

Contaminant analysis

Estrogenic substances were detected in river water with the T47D-Kbluc assay at all 8 sites, with concentrations ranging from 0.20 to 0.44 ng/L E2β-Eq (Supplemental Data, Table SI2). All concentrations detected were below the 2.0 ng/L predicted no-effect concentration (PNEC) for E2β (Caldwell et al. 2012). Of the 7 hormones assessed with PSDs, only ethinylestradiol was detected. It was detected at the 6 most downstream sites, and concentrations ranged from 0.27 to 1.62 ng/L, all in excess of the 0.1 ng/L PNEC for aquatic organisms (Caldwell et al. 2012). Two industrial contaminants, nonylphenol and BPA, were detected at all sites. The nonylphenol concentration ranged from 0.2 to 3.5 ng/L and was higher at downstream sites (>1 ng/L downstream of 74 Bridge). None of the concentrations measured exceeded the 6600 ng/L chronic exposure threshold determined by the US Environmental Protection Agency (2005). Concentrations of BPA ranged from 0.16 to 2.27 ng/L, and like nonylphenol, they were higher downstream (Supplemental Data, Table SI2). Concentrations of BPA did not exceed the 60 ng/L PNEC, determined by Wright-Walters et al. (2011) for the protection of aquatic organisms, at any site.

At all sites, PAHs were present in the water, and 31 of the 42 PAHs analyzed were detected. Total PAH concentrations ranged from 85.8 to 242.36 ng/L (Supplemental Data, Table SI2). Concentrations fluctuated among sites, with the highest levels occurring at the Route 801, Pee Dee, and Society Hill sites (Table 1). The ESBTU values ranged from 0.0009 to 0.035 toxic units for acute toxicity, all <1. For chronic toxicity, toxic units ranged from 0.017 to 0.071 toxic units, all <1, indicating low likelihood for any negative effects of water PAHs on aquatic life (Supplemental Data, Table SI3).

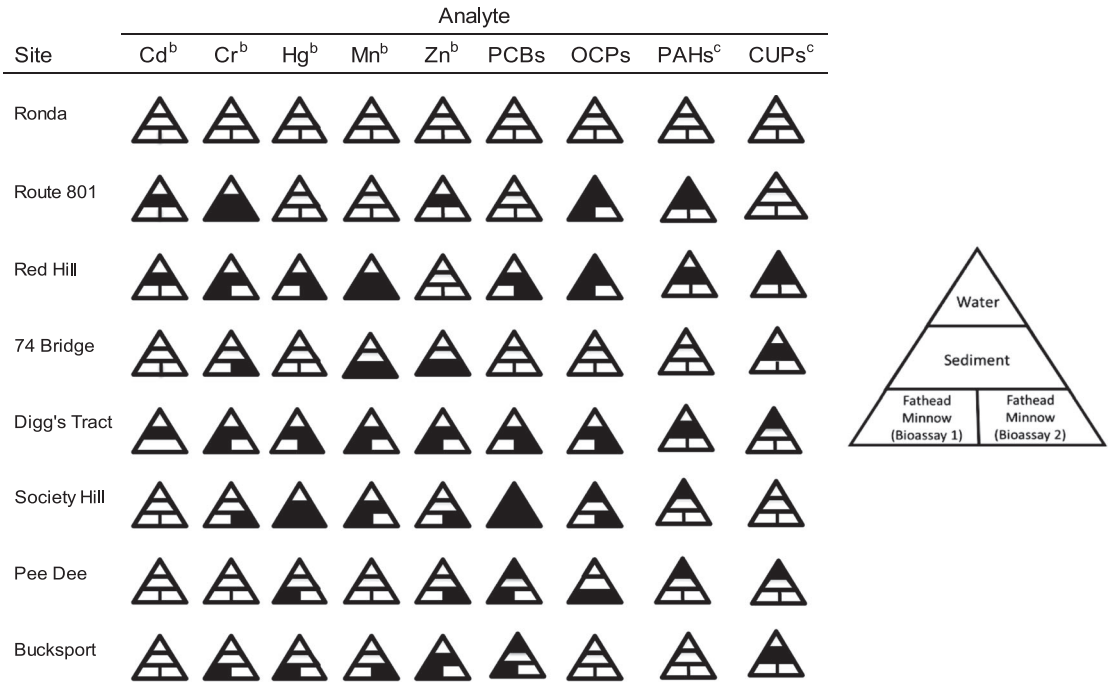
At all sites, OCPs were present in the water, and 9 of the 28 OCPs analyzed were detected. Total OCP concentrations ranged from 1.9 to 12.3 ng/L (Supplemental Data, Table SI2). No individual OCP concentration exceeded published thresholds. No combined OCP concentration threshold or aquatic life threshold exists. Concentrations of OCPs were noticeably elevated at the Red Hill, Route 801, and Society Hill sites (Table 1).

At all but one site (Ronda, most upstream site), PCBs were present in the water, and 9 of the 21 congeners analyzed were detected. Concentrations ranged from below detection limits (BDL) to 7.3 ng/L and were highest at the Pee Dee site (Supplemental Data, Table SI2; Table 1). Total PCB concentrations did not exceed the 14.0 ng/L chronic exposure threshold for freshwater aquatic life at any of the sites (US Environmental Protection Agency 2016).

At all sites, CUPs were present in the water, and 8 of the 47 CUPs analyzed were detected. Concentrations ranged from 1.4 to 190.7 ng/L and were highest at the Pee Dee, Red Hill, and Digg's Tract sites (Supplemental Data, Table SI2; Table 1). No CUPs exceeded known thresholds, among those published.

At all sites, PAHs were present in the sediment, and 37 of the 42 PAHs analyzed were detected. Total PAH concentrations ranged from 211 to 2960 ng/g dry weight and were

TABLE 1: Summary of selected contaminants among riverine sites during in situ bioassays[a]



[a]For each triangle, a filled section represents a measured contaminant concentration among the highest 3 for a given analyte at all sites (i.e., darker triangles indicate more frequently detected, high levels of contaminants; light triangles indicate contaminants less frequently detected, at lower levels).
[b]Metals not measured in water.
[c]Not measured in fish tissue.
PCB = polychlorinated biphenyl; OCP = organochlorine pesticide; PAH = polycyclic aromatic hydrocarbon; CUP = current-use pesticide.

highest at the Route 801, Digg's Tract, and Red Hill sites (Supplemental Data, Table SI2; Table 1). Total PAH levels exceeded the 1610 ng/g dry weight threshold-effect concentration for freshwater ecosystems (MacDonald et al. 2000) at the Route 801 site. Levels of PAHs did not exceed the PEC of 22 800 ng/g dry weight (MacDonald et al. 2000) at any site. The ESBTU values ranged from 0.004 to 0.069 for acute toxicity and from 0.015 to 0.281 for chronic toxicity (Supplemental Data, Table SI4). No sediment toxic units exceeded 1, indicating little likelihood of negative sediment PAH effects on aquatic life.

At 6 of the 8 sites, OCPs were detected in the sediment; they were not present at Ronda, the most upstream site, and Bucksport, the most downstream site. Four of the 28 OCPs analyzed were detected. Concentrations ranged from BDL to 2.8 ng/g dry weight and were highest at the Route 801, Digg's Tract, and Red Hill sites (Supplemental Data, Table SI2; Table 1). The most frequently detected OCP was 4,4′-dichlorodiphenyldichloroethylene (4,4′-DDE) with detections at 6 of the 8 sites. Concentrations of 4,4'-DDE did not exceed the threshold of 3.16 ng/g dry weight for freshwater ecosystems at any site (MacDonald et al. 2000). Chlordane was also detected at 2 sites (Route 801 and Digg's Tract) but did not exceed the 4.5 ng/g dry weight interim sediment quality guideline that corresponds to threshold-level effects (Environment Canada 1999).

At 4 of the 8 sites, PCBs were detected in the sediment, and 6 of the 21 congeners analyzed were detected. All congeners were summed to calculate a total PCB concentration, which ranged from BDL to 5.2 ng/g dry weight (Supplemental Data, Table SI2) and did not exceed the 59.8 ng/g dry weight threshold (MacDonald et al. 2000). Concentrations of PCB were highest at the Digg's Tract, Red Hill, and Society Hill sites (Table 1).

Atrazine was the only CUP detected in sediment and was found at 3 of the 8 sites. Concentrations were 3.8 ng/g dry weight at Red Hill, 3.4 ng/g dry weight at Bucksport, and 0.9 ng/g dry weight at the 74 Bridge site (Supplemental Data, Table SI2; Table 1). Atrazine concentrations did not exceed the 6.62 ng/g dry weight screening benchmark developed by the US Environmental Protection Agency (2006).

Sediment metals were detected at each site, and specific metals and concentrations varied among sites. Eighteen of the 22 metals analyzed were detected. Many metals did not exceed published thresholds, or thresholds do not exist; and their concentrations can be found in Supplemental Data, Table SI2. Selected metals known for their endocrine-disrupting capabilities are highlighted in Table 1. Manganese (Mn) exceeded the lowest effect level of 460 µg/g dry weight at 4 of the 8 sites and exceeded the severe effect level of 1100 µg/g dry weight at one site, Digg's Tract (Persaud et al. 1993).

One composite fathead minnow whole-body tissue sample was analyzed for contaminants from each site, for each bioassay. The baseline concentration of each contaminant was determined by averaging the 6 baseline fathead minnow sample concentrations. Fourteen of the 21 PCB congeners analyzed were detected in the fathead minnow tissue samples.

Baseline average total PCB concentration was 29.6 ng/g dry weight. Total PCB concentrations in fathead minnows surviving to the end of the bioassays were similar and ranged from 39.7 to 93.4 ng/g dry weight for fish of the first bioassay and from 34.6 to 92.0 ng/g dry weight for those in the second bioassay (Figure 5; Supplemental Data, Table SI2). All fathead minnow PCB concentrations were greater in the river-exposed fish when compared to baseline fish. No PCB action levels or thresholds were exceeded in fathead minnow tissue.

At every site, OCPs were detected in fathead minnow tissue samples. Seven of the 28 OCPs analyzed were detected, and mean baseline concentration was 31.6 ng/g dry weight. Total OCP concentrations for the first bioassay ranged from 42.3 to 66.1 ng/g dry weight and from 19.9 to 64.5 ng/g dry weight for the second bioassay (Supplemental Data, Table SI2). Except for 2 sites in the second bioassay, all riverine fathead minnow samples had higher OCP concentrations than baseline fathead minnow samples (Figure 5).

Seventeen of the 22 metals analyzed were detected in fish tissue samples. Concentrations of each metal varied, and few exceeded published thresholds available (Supplemental Data, Table SI2). Mercury was evaluated because of its known endocrine-disrupting capabilities (Georgescu et al. 2011), and fathead minnow baseline Hg concentration averaged 0.19 µg/g dry weight. Concentrations of Hg in fathead minnow from the first bioassay ranged from 0.25 to 0.38 µg/g dry weight and from 0.17 to 0.63 µg/g dry weight from the second bioassay (Figure 6). Except for one sample, Hg concentrations in fathead minnow increased in every river-exposed fish sample. Manganese was also examined because of its high rate of accumulation in fish tissue, and the fathead minnow Mn baseline average concentration was 4.5 µg/g dry weight. Concentrations of Mn in fathead minnow from the first bioassay ranged from 8.2 to 61.0 µg/g dry weight and from 15.4 to 49.1 µg/g dry weight from the second bioassay (Supplemental Data, Table SI2; Figure 6). Concentrations of Mn in all river-exposed fathead minnow samples exceeded concentrations in baseline samples.

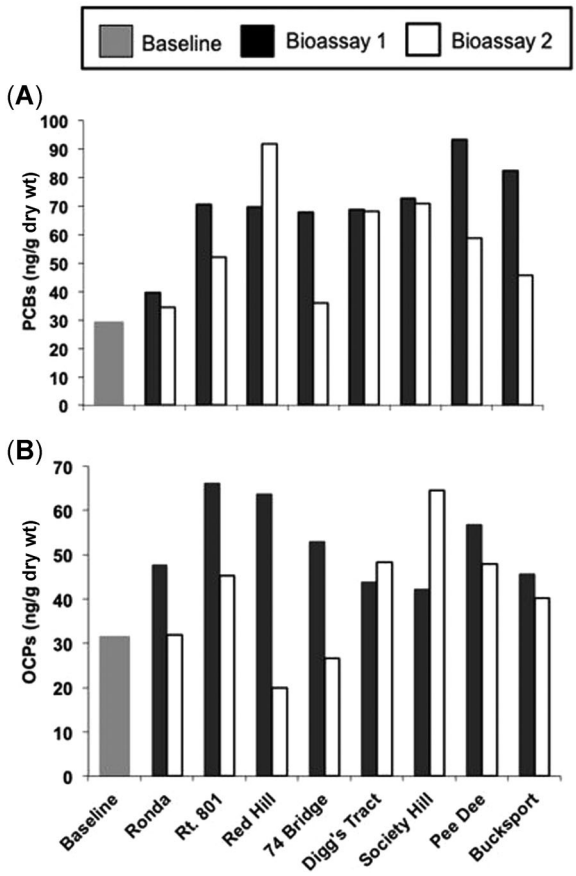


FIGURE 5: Polychlorinated biphenyl (**A**) and organochlorine pesticide (**B**) fathead minnow (*Pimephales promelas*) whole-fish concentrations (composite samples) from each site following in situ bioassays (1 and 2) and baseline samples. PCB = polychlorinated biphenyl; OCP = organo chlorine pesticide.

Water physicochemical characteristics

In general, water temperatures (Celsius) were warmer at downstream sites within each bioassay but were similar between bioassays (Table 2). Conductivity (microsiemens per centimeter) was also higher downstream and similar between bioassays. Salinity (parts per thousand) remained consistent and was slightly higher downstream. The dissolved oxygen level was higher at upstream sites and during the first bioassay. Values of pH were consistent between bioassays and similar among sites, with Bucksport, the most downstream site, exhibiting the lowest pH. At the Watha State Fish Hatchery pond with the largemouth bass cages, temperatures were slightly higher than those of the river, conductivity was more than double that of any river site, salinity was more than double that of any river site, and dissolved oxygen was higher (Table 2). At the McKinney Lake State Fish Hatchery pond with the robust redhorse cages, temperatures were higher, conductivity was one-half of any river site, salinity was low, dissolved oxygen was higher than at any river site, and pH was also higher than at any river site (Table 2). All dissolved oxygen and pH levels measured were suitable to support fish survival.

Fish survival correlations

Pairwise rank correlations were examined between fathead minnow survival and contaminants and water physicochemical characteristics. In the first bioassay, no strong ($p < 0.05$) correlations were found between survival and any contaminant or water variables. In the second bioassay, survival of fathead minnow was correlated with total PAHs (water, nanograms per liter; $p < 0.0001$, $r_s = 0.9701$), indicating a strong, positive, monotonic relationship. This result, however, was influenced by an outlier value and small sample size.

DISCUSSION

We examined the effects of contaminants on survival and health of caged fish in 2 longitudinal riverine bioassays, and the results indicated that water and sediment quality may have adversely affected the survival of robust redhorse, largemouth bass, and fathead minnow. A combined average of 43% of

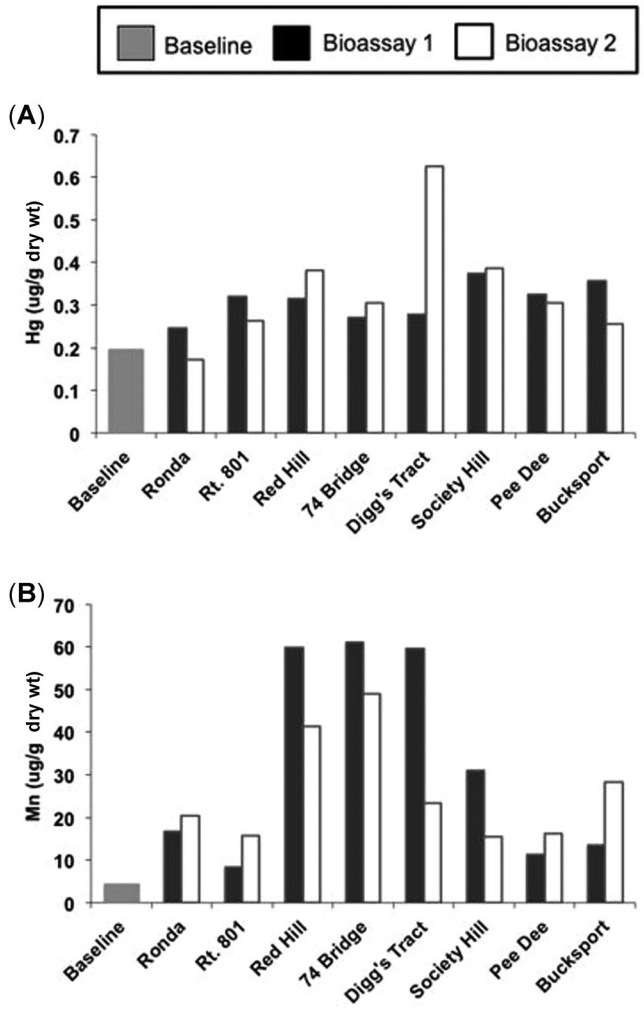


FIGURE 6: Mercury (**A**) and manganese (**B**) fathead minnow (*Pimephales promelas*) whole-fish concentrations (composite samples) from each site following in situ bioassays (1 and 2) and baseline samples.

fathead minnow survived the 28-d bioassays, but no robust redhorse or largemouth bass survived to the end of the bioassays. Fathead minnow survival among sites followed similar spatial patterns between the 2 bioassays, suggesting a consistent site-specific effect on survival. Ronda, the most upstream site, yielded high rates of mortality during both bioassays. Largemouth bass survival was slightly less than that of robust redhorse, which may have been influenced by the smaller size of the largemouth bass exposed or their sensitivity to riverine contaminants and water quality. Although hatchery ponds were aerated and this may have led to less stressful conditions than those experienced in the riverine environment, the survival in these ponds indicates that fish were able to survive in cages. Fathead minnow survival was significantly negatively correlated with water temperature and conductivity. Fluctuations in temperature have been shown to cause mortality, reduced growth, and the development of skin ulcers in fish; and conductivity fluctuations may indicate effluent discharges (Coulter et al. 2015). This correlative association may warrant additional investigation but does not necessarily imply a causal relationship.

Measured contaminant concentrations were not significantly correlated with fish survival among river sites, but the Digg's Tract site yielded the lowest survival of all fish species in both bioassays and generally had consistently high contaminant concentrations in the sediment, water, and fish tissue. Contaminants were present at every site, to varying degrees, and may have caused toxicant-induced stress to the fish. Previous studies have shown that when fathead minnow are exposed to contaminated effluent there are impacts on individual swimming performance and aerobic energy metabolism (Goertzen et al. 2011). Metals in effluent have also been linked to reduced reproductive success, significant metal accumulation into fish tissues, and overall metal toxicity (Rozon-Ramilo et al. 2011). Many contaminants detected did not exceed aquatic life thresholds; however, these thresholds, and our ability to measure contaminants, did not account for the possibility of toxicity from pulse exposures (high concentrations for short periods of time, which are difficult to measure), which may be associated with greater risk than continuous exposures (Diamond et al. 2005).

Accumulation of contaminants in fish tissue over the short duration of the bioassay (<28 d) was substantial. Concentrations of OCPs in fathead minnow tissue increased from baseline in all but 2 samples. Concentrations of PCBs increased at all sites, and although exposure was not significantly correlated with survival in the present study, reproduction and survival have been shown to be impacted by PCBs in fathead minnow when exposed for longer periods of time (Nebeker et al. 1974). Concentrations of Mn and Hg in fish tissue increased at almost every site. The toxicity of Mn and Hg, as well as the potential toxicity of other metals detected and their ability to act in an additive response (Norwood et al. 2003), may have contributed to mortality. Additional research to examine the effects of heavy metal accumulation on fish survival would be required to elucidate biotic responses.

Evidence revealed that fathead minnow were feeding within the cages, and weights of surviving fathead minnow were significantly reduced in bioassay individuals at almost every site at the end of the exposures, relative to baseline samples. In addition to water quality stress, this weight loss may have been partially due to inadequate food resources in the cages or greater energy expenditures in flowing river water (compared to relatively static hatchery ponds). Fathead minnow are known to be tolerant to low dissolved oxygen, high turbidity, and a variety of environmental conditions; however, they most typically occur in slow-moving water such as those found in river eddies and backwater areas and may have lower survival in fast-moving rivers, such as the Yadkin-Pee Dee River in the present study (Rohde et al. 2009). Nonetheless, the evidence of fathead minnow feeding (material in the GI tracts) and the accumulation of biofilm within the cages ultimately suggest that starvation alone was an unlikely primary factor in fathead minnow mortality. Whether this was also the case for largemouth bass and robust redhorse mortality is uncertain. There is a possibility that the behavior and physiological response of these species differ from those of fathead minnow and may have led to their mortality. The higher percentages of survival of robust

TABLE 2: Mean physicochemical characteristics of river water (standard error in parentheses) measured at each site during the 28-d bioassay

	Bioassay 1					Bioassay 2				
Site	Temperature (°C)	Conductivity (µS/cm)	Salinity (ppt)	Dissolved oxygen (mg/L)	pH	Temperature (°C)	Conductivity (µS/cm)	Salinity (ppt)	Dissolved oxygen (mg/L)	pH
Ronda	22.3 (0.5)	61 (2)	0.03 (0.00)	7.6 (0.2)	7.4 (0.0)	22.1 (0.3)	66 (3)	0.03 (0.00)	6.0 (0.7)	7.1 (0.1)
Route 801	25.7 (0.6)	83 (2)	0.04 (0.00)	7.2 (0.2)	7.4 (0.0)	24.9 (0.6)	94 (6)	0.04 (0.00)	5.9 (0.6)	7.3 (0.1)
Red Hill	25.5 (0.8)	99 (3)	0.04 (0.00)	5.2 (0.2)	7.2 (0.0)	26.3 (0.7)	118 (15)	0.05 (0.01)	5.0 (0.6)	7.1 (0.1)
74 Bridge	26.7 (0.8)	97 (3)	0.04 (0.00)	5.7 (0.3)	7.2 (0.0)	27.5 (0.4)	100 (4)	0.05 (0.00)	5.1 (0.3)	7.2 (0.1)
Diggs Tract	27.6 (0.4)	96 (1)	0.04 (0.00)	5.6 (0.5)	7.3 (0.0)	27.1 (0.4)	96 (8)	0.04 (0.00)	4.7 (0.4)	7.1 (0.1)
Society Hill	27.4 (0.9)	111 (11)	0.05 (0.00)	6.5 (0.3)	6.9 (0.0)	27.5 (0.3)	113 (5)	0.05 (0.00)	6.2 (0.3)	7.2 (0.1)
Pee Dee	27.7 (0.7)	106 (4)	0.05 (0.00)	6.1 (0.2)	7.0 (0.0)	27.6 (0.4)	113 (4)	0.05 (0.00)	5.5 (0.7)	7.2 (0.1)
Bucksport	27.8 (2.5)	97 (0)	0.05 (0.01)	5.1 (0.3)	6.7 (0.0)	27.1 (0.4)	96 (3)	0.04 (0.00)	4.7 (0.4)	6.8 (0.1)
Watha State Fish Hatchery	28.2 (0.6)	278 (2)	0.13 (0.00)	8.6 (1.1)	—	—	—	—	—	—
McKinney Lake Fish Hatchery	—	—	—	—	—	28.2 (0.7)	31 (1)	0.01 (0.0)	7.4 (0.1)	8.4 (0.4)

redhorse and largemouth bass in cages in hatchery ponds and positive evidence of feeding occurring when inside hatchery reference cages suggest that they could have fed and survived similarly in the cages in the river had contaminant and water quality conditions been suitable. In situ bioassays have been used with fathead minnows, and a number of other species, in a variety of conditions including nearby large rivers such as the Cape Fear and Roanoke Rivers (Hewitt et al. 2006; Cope et al. 2011) to yield findings informative to conservation. Hewitt et al. (2006) deployed Cape Fear shiners (*Notropis mekistocholas*) for 28 d at 10 riverine sites in the same types of cages that we used in the present study. Their research determined that average survival was 76% and ranged from 53 to 100%. Likewise, Cope et al. (2011) conducted in situ bioassays using both shortnose sturgeon (*Acipenser brevirostrum*) and fathead minnows deployed simultaneously in the same type of cages that we used for 22 d at 8 riverine sites. In their study, fathead minnow survival averaged 99.8%, whereas shortnose sturgeon survival was, on average, 9% for the same riverine sites. These studies demonstrate the utility of in situ bioassays with fish in these types of cages for assessing cumulative environmental conditions, that supplemental feeding is not required, and that the variability in species sensitivities and in site-specific riverine conditions influences survival of test species of conservation concern. Moreover, Sellin et al. (2009) conducted a caged fish study to investigate the impacts of wastewater-treatment plant effluent and found that the deployed fathead minnows were significantly affected by estrogens in surface water in as few as 7 d. That shorter study duration, compared to our 28-d bioassay, may warrant consideration for future related research. Simmons et al. (2017) conducted longer bioassays (21 d) with goldfish (*Carassius auratus*) and fed fish weekly. Miller et al. (2011) fed fathead minnows in a caged study using frozen brine shrimp (*Artemia* sp.) wrapped with nylon mesh and were able to maintain fish in cages for 6 wk. However, in the Miller et al. (2011) study, cages were in one nearby location, whereas our sites spanned approximately 550 km of the 700-km river. Although feeding test fish was done in these and other studies, it was not feasible in the present study because of logistical constraints and the fact that we did not want potential contaminants in supplied artificial food to influence our tissue analyses. Depending on research objectives, feeding may be an important consideration in future in situ bioassays for increasing the longevity and growth of fish. Miller et al. (2011) also assessed postcaging reproduction and offspring viability, and these parameters could be assessed in the Yadkin-Pee Dee River system during future research.

The intersex condition was not observed in any assayed fathead minnow, robust redhorse, or largemouth bass; but it was not expected in the juvenile robust redhorse or largemouth bass because of their sexual immaturity and absence of developed gonads. After approximately 3 yr of estrogen exposure, Kidd et al. (2007) found the intersex condition in fathead minnow males, demonstrating that prolonged exposure to synthetic estrogens causes intersex in fathead minnow. The absence of intersex in fathead minnow in the present study may have been due to low concentrations of contaminants, the short duration of exposure (28 d), the temporal window of exposure (fish were past the early life stages of gonadal development), or other unknown factors. Although sex was not determined for all fish used in this bioassay, there was a general reduction in presence and size of male nuptial tubercles in those fathead minnow exhibiting them after exposure to the river. This loss of nuptial tubercles was likely due to the stress of occupancy within a caged environment but may also have been a possible indicator of endocrine effects and exposure to estrogenic contaminants (Vajda et al. 2011).

Low survival rates of caged fish and the accumulation of contaminants into fish tissue over the bioassay period, as well as high associated sediment and water contaminant concentrations, indicate that pollutants may be exerting an adverse impact on juvenile and adult fish within the Yadkin-Pee Dee River. The complete mortality of juvenile largemouth bass and robust redhorse in the riverine exposures may indicate a critical recruitment bottleneck in these (and perhaps other) species at early life stages (Schiemer et al. 2003; Humphries et al. 2013), which may directly reflect the low population size, limited recruitment, and imperiled status of robust redhorse in the river. Contaminants accumulated within 28 d in fathead minnow tissues, indicating that contaminants may pose a substantial risk to fish health in this system, especially considering that wild fish are chronically exposed to ambient river conditions during all life stages, including those where critical reproductive changes occur.

A suite of chemical contaminants occurs in riverine ecosystems that we were unable to measure. The Yadkin-Pee Dee River ecosystem is known to contain a pervasive load of per- and polyfluoroalkyl substances that are unregulated but implicated for their persistence, bioaccumulative potential, toxicity, and global distribution (Penland et al. 2020). Cumulative stressors other than those we measured likely contributed to the fish mortality that we observed in these in situ bioassays. In the future, effective management actions should be implemented to improve water quality conditions and reduce contaminant influx within the river system. Improved wastewater-treatment technologies, reduced discharges from industrial facilities, best practices to reduce agricultural and urban runoff, and enhancing public awareness of pollutant impacts on the environment may be beneficial first steps. The present study provides important findings that may inform and help guide those actions toward maintaining and enhancing habitat for aquatic life.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5104.

Acknowledgment—The present study was funded by the North Carolina Wildlife Resources Commission through Federal Aid in Sport Fish Restoration Grant (NC-F-99-R) and by the North Carolina Wildlife Resources Commission and the South Carolina Department of Natural Resources through a competitive State Wildlife Grant (NC-U2-F14AP00075). We thank J. Wehbie, S. Gardner, B. Cope, A. Escobar, J. Archambault, C.

Lee Pow Jackson, J. Fischer, P. Lazaro, J. Leonard, S. Gadi, D. Shea, F. Weber, B. Albanese, and hatchery personnel for their assistance with fish production, field sampling, laboratory processing, data analyses, and manuscript review. The present study was performed under the auspices of North Carolina State University protocol 14-064-O.

Disclaimer—The North Carolina Cooperative Fish and Wildlife Research Unit is jointly supported by North Carolina State University, the North Carolina Wildlife Resources Commission, the US Geological Survey, the US Fish and Wildlife Service, and the Wildlife Management Institute. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US government.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (casey.grieshaber@gmail.com).

REFERENCES

ASTM International. 2007. Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. E 729-96. In *Annual Book of ASTM Standards*, Vol 11.06. Philadelphia, PA, USA.

Bahamonde PA, Munkittrick KR, Martyniuk CJ. 2013. Intersex in teleost fish: Are we distinguishing endocrine disruption from natural phenomena? *Gen Comp Endocrinol* 192:25–35.

Barton HA, Andersen ME. 1998. Endocrine active compounds: From biology to dose response assessment. *Crit Rev Toxicol* 28:363–423.

Blazer VS, Iwanowicz LR, Henderson H, Mazik PM, Jenkins JA, Alvarez DA, Young JA. 2012. Reproductive endocrine disruption in smallmouth bass (*Micropterus dolomieu*) in the Potomac River basin: Spatial and temporal comparisons of biological effects. *Environ Monit Assess* 184:4309–4334.

Caldwell DJ, Mastrocco F, Anderson PD, Länge R, Sumpter JP. 2012. Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol, and 17α-ethinylestradiol. *Environ Toxicol Chem* 31:1396–1406.

Colman JR, Baldwin D, Johnson LL, Scholz NL. 2009. Effects of the synthetic estrogen, 17α-ethinylestradiol, on aggression and courtship behavior in male zebrafish (*Danio rerio*). *Aquat Toxicol* 91:346–354.

Cope WG, Holliman FM, Kwak TJ, Oakley NC, Lazaro PR, Shea D, Augspurger T, Law JM, Henne JP, Ware KM. 2011. Assessing water quality suitability for shortnose sturgeon in the Roanoke River, North Carolina, USA with an in situ bioassay approach. *J Appl Ichthyol* 27:1–12.

Coulter DP, Sepúlveda MS, Troy CD, Höök TO. 2015. Species-specific effects of subdaily temperature fluctuations on consumption, growth and stress responses in two physiologically similar fish species. *Ecol Freshw Fish* 25:465–475.

Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environ Health Perspect* 107(Suppl. 6):907–938.

Diamond J, Bowersox M, Latimer H, Barbour C, Bearr J, Butcher J. 2005. Effects of pulsed contaminant exposures on early life stages of the fathead minnow. *Arch Environ Contam Toxicol* 49:511–519.

Dwyer FJ, Hardesty DK, Henke CE, Ingersoll CG, Whites DW, Augspurger T, Canfield TJ, Mount DR, Mayer FL. 2005. Assessing contaminant sensitivity of endangered and threatened aquatic species: Part III. Effluent toxicity tests. *Environ Contam Toxicol* 48:174–183.

Environment Canada. 1999. Canadian sediment quality guidelines for the protection of aquatic life—Chlordane. Canadian environmental quality guidelines. Canadian Council of Ministers of the Environment, Ontario, Canada.

Fisk JM, Kwak TJ, Heise RJ. 2015. Effects of regulated river flows on habitat suitability for the robust redhorse. *Trans Am Fish Soc* 144:792–806.

Georgescu B, Georgescu C, Dărăban S, Bouaru A, Paşcalău S. 2011. Heavy metals acting as endocrine disrupters. *Scientific Papers Animal Science and Biotechnologies* 44:89–93.

Goertzen MM, Driessnack MK, Janz DM, Weber LP. 2011. Swimming performance and energy homeostasis in juvenile laboratory raised fathead minnow (*Pimephales promelas*) exposed to uranium mill effluent. *Comp Biochem Physiol C Toxicol Pharmacol* 154:420–426.

Goulden CE. 1999. Bioassays: A critique of effluent bioassays. In Schultze PE, ed, *Measures of Environmental Performance and Ecosystem Condition*. National Academy Press, Washington, DC, USA, pp 191–198.

Grieshaber CA, Penland TN, Kwak TJ, Cope WG, Heise RJ, Law JM, Shea D, Aday DD, Rice JA, Kullman SW. 2018. Relation of contaminants to fish intersex in riverine sport fishes. *Sci Total Environ* 643:73–89.

Hewitt AH, Cope WG, Kwak TJ, Augspurger T, Lazaro PR, Shea D. 2006. Influence of water quality and associated contaminants on survival and growth of the endangered Cape Fear shiner (*Notropis mekistocholas*). *Environ Toxicol Chem* 25:2288–2298.

Hinck JE, Blazer VS, Schmitt CJ, Papoulias DM, Tillitt DE. 2009. Widespread occurrence of intersex in black basses (*Micropterus* spp.) from U.S. rivers, 1995–2004. *Aquat Toxicol* 95:60–70.

Humphries P, Richardson A, Wilson G, Ellison T. 2013. River regulation and recruitment in a protracted-spawning riverine fish. *Ecol Appl* 23: 208–225.

Kaplan EL, Meier P. 1958. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 53:457–481.

Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW. 2007. Collapse of a fish population after exposure to a synthetic estrogen. *Proc Natl Acad Sci USA* 104:8897–8901.

Kwak TJ, Freeman MC. 2010. Assessment and management of ecological integrity. In Hubert WA, Quist MC, eds, *Inland Fisheries Management in North America*, 3rd ed. American Fisheries Society, Bethesda, MD, pp 353–394.

Lee Pow CSD, Law JM, Kwak TJ, Cope WG, Rice JA, Kullman SW, Aday DD. 2017a. Endocrine active contaminants in aquatic systems and intersex in common sport fish. *Environ Toxicol Chem* 36:959–968.

Lee Pow CSD, Tilahun K, Creech K, Law JM, Cope WG, Kwak TJ, Rice JA, Aday DD, Kullman SW. 2017b. Windows of susceptibility and consequences of early life exposures to 17β-estradiol on medaka (*Oryzias latipes*) reproductive success. *Environ Sci Technol* 51:5296–5305.

MacDonald DD, Ingersoll CG, Berger TA. 2000. Development and evaluation of consensus-based sediment quality guidelines for freshwater ecosystems. *Arch Environ Contam Toxicol* 39:20–31.

Miller JL, Sherry J, Parrott J, Quinn JS. 2011. A subchronic in situ exposure method for evaluating effects in small-bodied fish at contaminant sites. *Environ Toxicol* 29:54–63.

Muthumbi W, De Boever P, Pieters JG, Siciliano S, D'Hooge W, Verstraete W. 2003. Polycyclic aromatic hydrocarbons (PAHs) and estrogenic compounds in experimental flue gas streams. *J Environ Qual* 32:417–422.

Nebeker AV, Puglisi FA, DeFoe DL. 1974. Effect of polychlorinated biphenyl compounds on survival and reproduction of the fathead minnow and flagfish. *Trans Am Fish Soc* 103:562–568.

Nolan M, Jobling S, Brighty G, Sumpter JP, Tyler CR. 2001. A histological description of intersexuality in the roach. *J Fish Biol* 58:160–176.

North Carolina Wildlife Resources Commission. 2019. Final performance report for aquatic wildlife diversity operations 2018–2019. Raleigh, NC, USA.

Norwood WP, Borgmann U, Dixon DG, Wallace A. 2003. Effects of metal mixtures on aquatic biota: A review of observations and methods. *Hum Ecol Risk Assess* 9:795–811.

Penland TN, Cope WG, Kwak TJ, Strynar MJ, Grieshaber CA, Heise RJ, Sessions FW. 2020. Trophodynamics of per- and polyfluoroalkyl substances in the food web of a large Atlantic slope river. *Environ Sci Technol* 54:6800–6811.

Penland TN, Grieshaber CA, Kwak TJ, Cope WG, Heise RJ, Sessions FW. 2018. Food web contaminant dynamics of a large Atlantic slope river: Implications for common and imperiled species. *Sci Total Environ* 633:1062–1077.

Persaud D, Jaagumagi R, Hayton A. 1993. Guidelines for the protection and management of aquatic sediment quality in Ontario. Ministry of Environment and Energy, Toronto, ON, Canada.

Robust Redhorse Conservation Committee. 2018. Electrofishing surveys for robust redhorse on the Pee Dee River, North and South Carolina. Yadkin-Pee Dee Technical Working Group, Raleigh, NC, USA.

Rohde FC, Arndt RG, Foltz JW, Quattro JM. 2009. *Freshwater Fishes of South Carolina*. University of South Carolina Press, Columbia, SC, USA, pp 170–172.

Rozon-Ramilo LD, Dubé MG, Squires AJ, Niyogi S. 2011. Examining waterborne and dietborne routes of exposure and their contribution to biological response patterns in fathead minnow (*Pimephales promelas*). *Aquat Toxicol* 105:466–481.

Sackett DK, Pow CL, Rubino MJ, Aday DD, Cope WG, Kullman S, Rice JA, Kwak TJ, Law JM. 2015. Sources of endocrine-disrupting compounds in North Carolina waterways: A geographic information systems approach. *Environ Toxicol Chem* 34:437–445.

Schiemer F, Keckeis H, Kamler E. 2003. The early life history stages of riverine fish: Ecophysiological and environmental bottlenecks. *Comp Biochem Physiol A Mol Integr Physiol* 133:439–449.

Scholz S, Mayer I. 2008. Molecular biomarkers of endocrine disruption in small model fish. *Mol Cell Endocrinol* 293:57–70.

Sellin MK, Snow DD, Akerly DL, Kolok AS. 2009. Estrogenic compounds downstream from three small cities in eastern Nebraska: Occurrence and biological effect. *J Am Water Resour Assoc* 45:14–21.

Simmons DBD, Miller J, Clarence S, McCallum ES, Balshine S, Chandramouli B, Cosgrove J, Sherry JP. 2017. Altered expression of metabolites and proteins in wild and caged fish exposed to wastewater effluents in situ. *Sci Rep* 7:17000.

Tan SW, Meiller JC, Mahaffey KR. 2009. The endocrine effects of mercury in humans and wildlife. *Crit Rev Toxicol* 39:228–269.

US Environmental Protection Agency. 1996. Method 3050B—Acid digestion of sediments, sludges, and soils. Revision 2. Washington, DC.

US Environmental Protection Agency. 2005. Aquatic life ambient water quality criteria nonylphenol. EPA-822-R-05-005. Washington, DC. [cited 2016 June 6]. Available from: http://www.epa.gov/waterscience/criteria/aqlife.html

US Environmental Protection Agency. 2006. Freshwater sediment screening benchmarks. Region III BTAG. Washington, DC. [cited 2016 June 6]. Available from: http://www.epa.gov/risk/freshwater-sediment-screening-benchmarks

US Environmental Protection Agency. 2012. Equilibrium partitioning sediment benchmarks (ESBs) for the protection of benthic organisms: Procedures for the determination of the freely dissolved interstitial water concentrations of nonionic organics. EPA-600-R-02-012 65. Washington, DC.

US Environmental Protection Agency. 2016. National recommended water quality criteria—Aquatic life criteria table. Washington, DC. [cited 2016 June 6]. Available from: http://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table#table

Vajda AM, Barber LB, Gray JL, Lopez EM, Bolden AM, Schoenfuss HL, Norris DO. 2011. Demasculinization of male fish by wastewater treatment plant effluent. *Aquat Toxicol* 103:213–221.

Warren ML Jr, Burr, BM, Walsh SJ, Bart HLJ, Cashner RC, Etnier DA, Freeman BJ, Kuhajda BR, Mayden RL, Robison HW, Ross ST, Starnes WC. 2000. Diversity, distribution, and conservation status of the native freshwater fishes of the southern United States. *Fisheries* 25:7–31.

Wright-Walters M, Volz C, Talbott E, Davis D. 2011. An updated weight of evidence approach to the aquatic hazard assessment of bisphenol A and the derivation a new predicted no effect concentration (PNEC) using a non-parametric methodology. *Sci Total Environ* 409:676–685.

Yost EE, Meyer MT, Dietze JE, Williams CM, Worley-Davis L, Lee B, Kullman SW. 2014. Transport of steroid hormones, phytoestrogens, and estrogenic activity across a swine lagoon/sprayfield system. *Environ Sci Technol* 48:11600–11609.

