

ASSESSMENT OF HEAVY METAL AND PAH EXPOSURE IN LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*) IN THE REEDY RIVER WATERSHED, SOUTH CAROLINA, USA: A MULTI-SEASON ASSESSMENT OF METALLOTHIONEIN AND BILE FLUORESCENCE

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(Submitted 19 June 2012; Returned for Revision 29 July 2012; Accepted 7 August 2012)

Abstract—Biomarkers can be used as tools to help determine ecological exposure in watershed assessments. In the present study, metallothionein and fixed wavelength bile fluorescence for two-, four-, and five-ring hydrocarbons were used as biomarkers of exposure in largemouth bass (*Micropterus salmoides*) in the Reedy River watershed located in South Carolina, USA. Fish were sampled from three impoundments and a reference site over three seasons in the same year. Biomarker endpoints were compared to chemical concentrations at each site during each season. Results indicated that despite elevated concentrations of hydrocarbons being present in sediments, the hydrocarbons did not appear to be bioavailable based on bile fluorescence analysis. Bile fluorescence analysis also indicated that the hydrocarbons detected in this watershed were likely of petrogenic origin. Significantly elevated sediment concentrations of Cd, Cr, Cu, Pb, Ni, Ag, Zn, and metallothionein were found in one impoundment, Lake Conestee, compared with the reference site, indicating both the presence and bioavailability of these metals. Seasonal variability of bile fluorescence was limited; however, metallothionein showed elevated concentrations in the spring and summer compared with fall. Environ. Toxicol. Chem. 2012;31:2763–2770. © 2012 SETAC

Keywords—Biomarkers *Micropterus salmoides* Metallothionein Bile fluorescence Metals

INTRODUCTION

The Reedy River drains approximately 651 km² within the Southern Inner and Outer Piedmont ecoregions of the upper Santee River basin in northwestern South Carolina [1]. Two potentially significant sources of contamination are involved in the Reedy River: (1) urban runoff from the city of Greenville, South Carolina, USA, and (2) the lingering effects of a large oil spill that occurred in the Reedy River in June 1996. Since it was established along the Reedy River in the early 1800s, the City of Greenville has been known as a major industrial and textile production center. At the time of the present study, Greenville had a population of approximately 56,000 and was one of the fastest growing areas in South Carolina. The pipeline spill, which occurred 16 km downstream of Greenville on the Reedy River, released 22,800 barrels (3,624,910 L) of No. 2 fuel oil into the environment, resulting in near complete fish and macroinvertebrate extirpation [2]. Although both sources of pollution have likely impacted the Reedy River, few assessments have been performed to evaluate their impact [2,3].

Biochemical biomarkers can be used as tools to help determine ecological exposure in watershed assessments. They can help to identify the bioavailable fraction of particular contaminants present in an ecosystem and help bridge the gap between chemical concentrations observed at a specific field site and the actual bioavailable contaminant exposure that is occurring. Watershed studies using biomarkers have successfully shown the effects of multiple contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and heavy metals (i.e., Cd, Pb, As, and Cu) [4–8].

Polycyclic aromatic hydrocarbons and heavy metals are two classes of pollutants that are ubiquitous and have known deleterious effects on fish [9,10]. Polycyclic aromatic hydrocarbons are formed through the incomplete combustion of fossil fuels and organic material [11] and by biosynthesis by microbes and plants [10,12]. Some PAHs are known to possess mutagenic and carcinogenic properties [10,13]. Heavy metals are natural elements whose concentration and toxicity is heavily influenced by anthropogenic activities [9].

Bile fluorescence (BF) is a biomarker used to measure PAH exposure at contaminated field sites [14,15]. The detoxification of lipophilic xenobiotic compounds, such as PAHs, occurs by enhancing the water solubility of the compound, therefore aiding its elimination from the organism [16]. After PAHs are biotransformed in the liver, most metabolites are excreted into bile, concentrated, and stored in the gallbladder until the bile is emptied into the intestinal tract. This process allows the bile metabolites to be used as an indicator of PAH exposure through the measurement of BF [13,17,18]. Metallothioneins (MTs) are a family of low-molecular-mass, metal-binding proteins that can regulate essential metals (Cu and Zn) and detoxify nonessential metals (Cd and Hg) [9]. The role of MTs in sequestering metals is well established, and their induction by metal exposure is associated with protection against metal toxicity [19]. Several field studies with feral fish have demonstrated that increased MT levels were positively correlated with metal exposure [20–22].

The goal of the present study was to investigate the environmental exposure to PAHs and heavy metals in fish inhabiting the Reedy River watershed. The specific objectives of the present study were to: (1) investigate BF and MT concentrations in largemouth bass (*Micropterus salmoides*), along with PAH and metal concentrations in sediments from three sites in the Reedy River and one reference site; (2) characterize the seasonal variability of sediment metal concentrations,

All Supplemental Data may be found in the online version of this article.

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Published online 31 August 2012 in Wiley Online Library
(wileyonlinelibrary.com).

PAHs, and biomarkers; and (3) investigate the relationship between biomarker endpoints and contaminant concentrations at each site.

MATERIALS AND METHODS

Sampling sites

The focus area of this study was the Reedy River watershed, located south of Greenville, South Carolina, USA (Fig. 1). The Reedy River contains three manmade impoundments that were used as sampling locations: Lake Conestee, Boyd Mill Pond, and Lake Greenwood. As a reference, Lake Robinson, a relatively pristine impoundment located on the nearby South Tyger River in upper Greenville County, was chosen [3]. Lake Conestee is the first impoundment on the Reedy River downstream of the urbanized section of Greenville. Boyd Mill Pond, located approximately 40 km downstream of Lake Conestee, is the first impoundment downstream of the oil pipeline spill site and is surrounded by landscape dominated by forest and agriculture. Lake Greenwood, located approximately 30 km downstream of Boyd Mill Pond, is surrounded by forested and agriculture landscape as well. Overall, the land cover of this watershed is approximately 42% forest, 28% urban, 18% agriculture, 8% grassland/shrubland, and 3% wetland [2].

Fish collections

Seven adult largemouth bass (greater than 20 cm in total length) were collected by electrofishing at each site during three seasons in 2004: spring (May), summer (July–August), and fall (October–December). After collection, each fish was measured for total length (cm) and weight (g) and identified by sex. The gallbladder, liver, and a muscle filet (skinless and boneless) were collected from each fish. Gallbladders and muscle filets were stored at -20°C , livers were wrapped in foil, flash frozen in liquid nitrogen, and then stored at -80°C until analysis.

Sediment PAH analysis

Sediment PAH concentrations at each site during each season were measured during a previous study [3]. Briefly, superficial (top 8 cm) sediment samples were taken from locations corresponding to areas of fish collection at a water depth of approximately 1.5 m. Polycyclic aromatic hydrocarbon

concentrations were measured via enzyme-linked immunosorbent assay and expressed as the sum of phenanthrene, fluoranthene, benzo[*a*]pyrene, pyrene, chrysene, anthracene, indeno[1,2,3-*cd*]pyrene, heating fuel, JP-5, JP-4, gasoline, kerosene, jet A fuel, benzo[*a*]anthracene, fluorene, benzo[*b*]fluoranthene, acenaphthylene, benzo[*k*]fluoranthene, acenaphthalene, and benzo[*ghi*]perylene.

Metal analysis

One surface water sample and one superficial (top 8 cm) sediment sample were taken from three different locations at each site during each season (same locations as PAH samples). Water and sediment samples were placed in separate pre-clean acid-washed Nalgene plastic bottles and stored at 4°C until analysis.

Sediment samples were oven-dried at 100°C until all moisture was eliminated. Samples were then digested via microwave-assisted acid digestion using 6 M nitric acid and following U.S. Environmental Protection Agency (U.S. EPA) Method 3051a. All samples were filtered through a $0.45\text{-}\mu\text{m}$ nylon filter before analysis.

Muscle ($n = 7$; summer only) and liver ($n = 2$; summer only) samples from fish collected during summer were dried at 100°C until all moisture was eliminated, acidified in 6 M nitric acid using acid-assisted microwave digestion, and filtered through a $0.45\text{-}\mu\text{m}$ nylon filter.

Water samples collected during summer were filtered through a $0.45\text{-}\mu\text{m}$ nylon filter and acidified with 6 M nitric acid.

All samples (water, sediment, fish muscle, and liver) were analyzed according to U.S. EPA method 6020 for Cr, Ni, Cu, Zn, As, Se, Ag, Cd, and Pb by inductively coupled plasma mass spectrometry. Quality control procedures consisted of blanks and spikes for each metal analyzed. All metal concentrations that were below the detection limit are reported at the detection limit.

Preparation of liver cytosol fraction

All livers were homogenized, and cytosolic fractions were prepared by homogenizing approximately 2 g liver (Biospec Products, Tissue Tearor) in 10 ml stabilization buffer (0.25 M sucrose, 0.05 M Tris-base, 1 mM ethylenediaminetetra-acetic

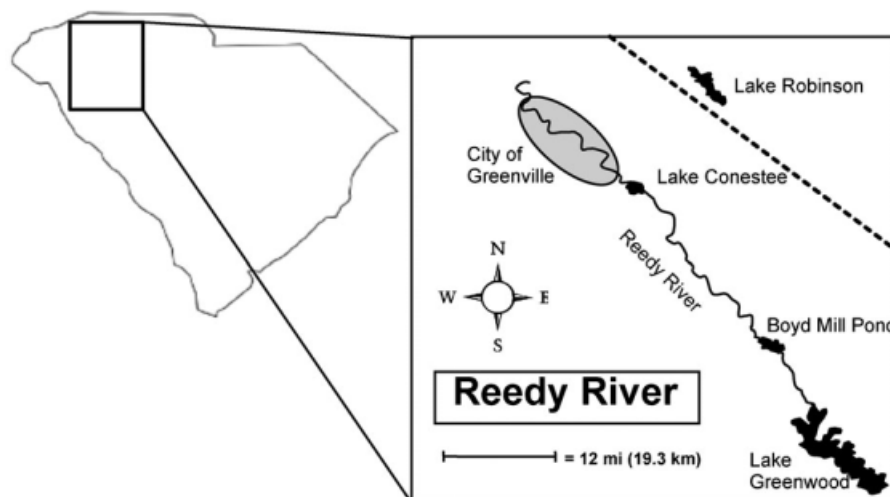


Fig. 1. Locations of sampling sites in South Carolina, USA. Dashed line indicates watershed boundary.

acid, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl-fluoride, pH 7.4). The homogenate was centrifuged for 20 min at 10,000 *g*, and the resulting supernatant (S9 fraction) collected and centrifuged for 65 min at 100,000 *g*. This supernatant (cytosolic fraction) was transferred to 2.0 ml microcentrifuge tubes (Fisher Scientific) and stored at -80°C until analysis. Cytosolic protein concentrations were measured using a colorimetric plate reader (Molecular Devices Spectramax 190) at 562 nm using a bicinchoninic acid protein assay kit with bovine serum albumin as a standard.

Metallothionein

Metallothionein was measured using the cytosolic fraction of the liver by a modification of the method of Viarengo et al. [23]. One milliliter of cytosol was mixed with 1.05 ml ice-cold ethanol and 80 μl chloroform. The mixture was centrifuged at 5,000 *g* for 15 min at 4°C . The supernatant was transferred to a new tube containing 40 μl of 37% HCl and 5 ml ice-cold ethanol, capped, and stored at -20°C for 1 h. Samples were centrifuged at 4,500 *g* for 15 min at 4°C . The supernatant was discarded, and 2 ml of 87% ethanol, 1% chloroform, and 12% homogenization buffer was added to the pellet and centrifuged at 4,500 *g* for 15 min at 4°C . Again the supernatant was discarded. The pellet was dried under nitrogen gas, after which it was resuspended in 150 μl of 0.25 NaCl and 150 μl of 1 N HCl containing 4 mM ethylenediaminetetra-acetic acid. Ellmans reagent (4 ml) was added to each sample. All samples were then centrifuged at 3,000 *g* for 5 min; 200 μl of each sample was pipetted in triplicate into a 96-well plate and read for absorbance at 412 nm (Molecular Devices Spectramax 190). A standard curve of glutathione was prepared at concentrations of approximately 1,200 μM to 75 μM . The conversion of glutathione to MT concentration was based on the ratio of 1 μM glutathione:0.055 μM MT as cited by Viarengo et al. [23]. All results were normalized to the amount of protein present in the cytosol.

Bile fluorescence

Gallbladders were thawed at room temperature in the dark and punctured using a scalpel. Bile was transferred to 1.5 ml microcentrifuge tubes (Fisher Scientific) and centrifuged for 5 min at 10,000 *g*. The supernatant was diluted 1:1,600 in 48% ethanol and measured using a Spectramax Gemini 96-well plate reader (Molecular Devices). Fixed wavelength fluorescence measurements were taken at 290/335, 341/383, and 380/430 nm for two-ring, four-ring, and five-ring PAHs, respectively [13]. Total bile protein was measured using a bicinchoninic acid protein assay kit with bovine serum albumin as a standard. Final BF values are reported as bile fluorescence intensity normalized to the total biliary protein. The mean of triplicate samples subtracted from blanks (48% ethanol) was used to achieve fluorescence intensity.

Statistical analysis

Sediment metal and PAH concentrations, MT, and BF values were investigated using two-way analysis of variance (ANOVA) with Tukey's post hoc tests (variables of site and season). Analysis of metal concentrations in fish muscle, fish liver, water, and analysis of sex differences in MT and BF values were performed via one-way ANOVA to determine significant differences based on site. All analyses were performed using JMP Pro 9 software. Significant differences for one- and two-way ANOVAs were defined by *p* values < 0.05 .

RESULTS

Sediment PAHs

No significant site-season interactions were found for sediment PAHs ($F_{6,24} = 1.12$; $p = 0.38$; Fig. 2), so the main effects of site and season were investigated. Results showed no significant effect of season ($F_{2,24} = 2.79$, $p = 0.081$), but a significant effect of site location ($F_{3,24} = 23.66$, $p < 0.001$) was observed. Post hoc testing showed a significant increase in PAH concentration in sediments at Lake Conestee compared with all sites, during each season. No other significant differences were observed between sites, except Lake Conestee, during all seasons.

Bile fluorescence

No significant differences in BF of two-ring ($F_{1,41} = 0.46$, $p = 0.50$), four-ring ($F_{1,32} = 0.27$, $p = 0.61$), or five-ring ($F_{1,41} = 1.17$, $p = 0.29$) PAH metabolites were found between sexes; therefore, data were combined from each site and season for further analysis (Fig. 3A–C). Significant site-season interactions were found for two-ring ($F_{5,34} = 3.53$, $p = 0.011$) and five-ring ($F_{5,34} = 5.97$, $p < 0.001$) PAH metabolites. No significant interaction effect was found for four-ring PAH metabolites ($F_{5,25} = 1.38$, $p = 0.26$), so the main effects of site and season were investigated. Significant differences based on season ($F_{1,25} = 7.32$, $p = 0.012$) but not site ($F_{2,25} = 1.92$, $p = 0.17$) were observed, with greater concentrations of four-ring PAH metabolites present in the summer compared with fall. Metabolite concentrations observed in the spring were not significantly different from those observed in either summer or fall. In spring and summer, only one bile sample was used for analysis at Lake Greenwood because of lack of bile being present in fish; therefore, for these two seasons Lake Greenwood was excluded from analyses.

Metals

For the nine metals analyzed (Cr, Ni, Cu, Zn, As, Se, Ag, Cd, Pb), no significant site-season interactions were observed (Supplemental Data) when comparing sediment concentrations; therefore, the main effects of site and season were investigated for each metal (Table 1). No significant effect of season was

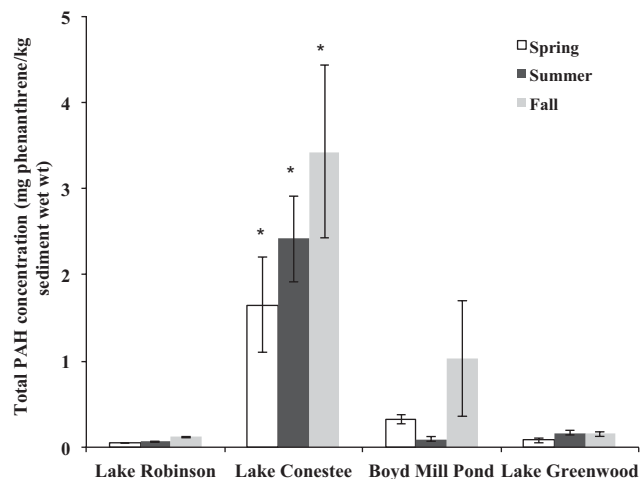


Fig. 2. Concentration of polycyclic aromatic hydrocarbons (PAHs) in the sediments of sampling sites. Significant differences between sites compared with Lake Robinson (reference) in the same season are indicated by * ($p < 0.05$). Data modified from Schreiber et al. [3].

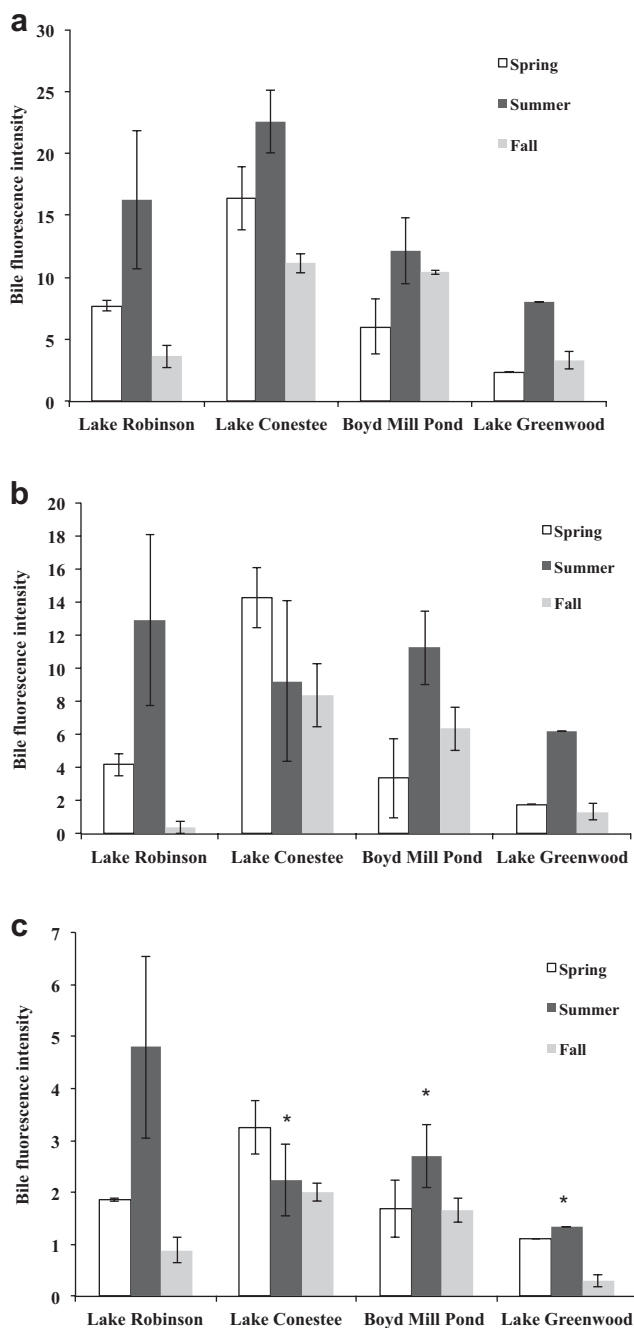


Fig. 3. (A) Two-ring, (B) four-ring, and (C) five-ring bile fluorescence intensities in largemouth bass. Significant differences between sites compared with Lake Robinson (reference) in the same season are indicated by * ($p < 0.05$; two-way analysis of variance). Data presented as mean \pm standard error mean (sample sizes range from 3–7).

seen for any of the nine metals analyzed (Supplemental Data). However, site location was shown to be a significant factor in each of the nine metals. Post hoc testing revealed that sediments from Lake Conestee had significantly greater concentrations of each metal than any other impoundment. No other significant differences in sediment metal concentrations, for any metal, were observed except those observed in Lake Conestee.

Analysis of metal concentrations in muscle filets and livers showed no significant difference between any site and the reference site, except arsenic muscle concentrations (Table 1; Supplemental Data). Mean muscle arsenic concentrations in muscle filets were significantly higher at Boyd Mill Pond

($p < 0.001$) and Lake Greenwood ($p = 0.018$) when compared with Lake Robinson.

Analysis of metal concentrations in water samples from summer found that all metals, except As, Cu, and Se, were below the detection limit, and no significant site differences existed for any metal.

Metallothionein

No significant differences in MT concentrations were found between sexes ($F_{1,73} = 2.62$, $p = 0.11$); therefore, data were combined from each site and season for further analysis (Fig. 4). No significant correlations existed between MT concentrations and fish length or fish weight (data not shown). No significant site-season interaction effect was found for MT; therefore, the main effects of site and season were investigated. Site was found to have a significant effect on MT concentrations ($F_{3,63} = 5.92$, $p = 0.001$), with both Lake Conestee ($p = 0.008$) and Boyd Mill Pond ($p = 0.005$) having significantly higher concentrations than those observed in Lake Robinson. A significant season effect was also observed ($F_{2,63} = 7.90$, $p < 0.001$), with spring ($p = 0.038$) and summer ($p < 0.001$) having higher average concentrations than observed in fall. Results from further post hoc analysis that investigated site differences within each season revealed that only during spring were significant differences observed. During spring, concentrations of MT in fish from both Lake Conestee ($p = 0.041$) and Boyd Mill Pond ($p = 0.033$) were significantly higher than those analyzed from Lake Robinson.

DISCUSSION

Sediment PAH analysis in the major impoundments within the Reedy River watershed revealed that Lake Conestee was the most impacted site for PAHs, having significantly higher concentrations than all other sites throughout the three sampling seasons of the present study [3] (Fig. 2). Elevated concentrations of PAHs have been linked to asphalt surfaces, car tires, and rainwater from impervious surfaces [24,25], all of which are associated with increases in urban land use. In a study examining aerial deposition of PAHs in multiple cities in the United Kingdom, Halsall et al. [11] observed the highest concentrations of PAHs in the most highly urbanized areas. Sediment PAH concentrations in the Reedy River indicate that the most likely source of PAHs is the Greenville urban area, and the trend of decreasing concentrations of sediment PAHs downstream of Lake Conestee implies a potential dilution of PAHs with increasing distance from the City of Greenville.

Diesel fuel oil No. 2, the type of oil introduced into the Reedy River during the 1996 oil spill, is known to contain many of the compounds included in the sediment analysis performed by Schreiber et al. [3,26,27]. No greater concentrations of sediment PAHs were observed at Boyd Mill Pond compared with Lake Conestee or Lake Robinson, indicating that remnants of this oil spill were either not present or not detected in Boyd Mill Pond sediments. This is significant because Boyd Mill Pond is the first impoundment downstream of where the oil spill occurred, and therefore, had the potential to act as a collection point for sediment-bound PAHs from the spill.

Bile fluorescence is a biomarker that has been used as a monitoring tool for PAH contamination, as well as an indicator of PAH source [13,17,28]. In the present study, BF analysis in largemouth bass for two-, four-, and five-ring PAHs showed no site-specific differences. These results were not expected given the significantly elevated concentrations of PAHs known to be

Table 1. Sediment, muscle, and liver metal concentrations^a

	Sediment ^b			Fish muscle ^c	Fish liver ^d
	Spring	Summer	Fall	Summer	Summer
Chromium					
Lake Robinson	10.7 ± 0.9	8.2 ± 1.2	17.3 ± 4.9	0.1 ± 0.0	0.0 ± 0.0
Lake Conestee	176.2 ± 92.8	74.5 ± 4.4	90.8 ± 9.4	0.1 ± 0.0	0.1 ± 0.1
Boyd Mill Pond	9.9 ± 6.7	15.3 ± 9.0	16.7 ± 8.4	0.1 ± 0.0	0.3 ± 0.3
Lake Greenwood	10.0 ± 3.9	10.2 ± 6.4	10.2 ± 0.8	0.1 ± 0.0	0.1 ± 0.1
Detection limit			0.03 mg/kg dry wt		
Nickel					
Lake Robinson	2.7 ± 0.5	2.3 ± 0.5	2.7 ± 0.6	0.1 ± 0.0	0.1 ± 0.0
Lake Conestee	11.7 ± 1.3	28.5 ± 2.1	14.0 ± 1.0	0.1 ± 0.0	0.2 ± 0.0
Boyd Mill Pond	2.1 ± 1.2	5.8 ± 5.0	3.1 ± 1.4	0.1 ± 0.1	0.2 ± 0.1
Lake Greenwood	2.7 ± 1.4	5.3 ± 4.5	2.3 ± 0.6	0.7 ± 0.2	0.2 ± 0.0
Detection limit			0.05 mg/kg dry wt		
Copper					
Lake Robinson	15.8 ± 2.1	2.7 ± 0.8	10.5 ± 2.9	0.7 ± 0.1	8.9 ± 0.0
Lake Conestee	46.1 ± 3.0	47.0 ± 5.6	57.2 ± 10.1	1.0 ± 0.2	48.6 ± 2.7
Boyd Mill Pond	4.3 ± 2.7	4.3 ± 1.9	5.2 ± 2.3	0.9 ± 0.1	6.5 ± 2.2
Lake Greenwood	4.0 ± 1.7	4.7 ± 3.1	4.1 ± 1.3	0.6 ± 0.1	26.8 ± 13.8
Detection limit			0.06 mg/kg dry wt		
Zinc					
Lake Robinson	18.2 ± 3.4	15.4 ± 0.9	20.3 ± 6.3	11.6 ± 0.8	53.1 ± 3.7
Lake Conestee	195.4 ± 82.4	367.1 ± 56.2	223.7 ± 78.1	13.3 ± 1.4	87.3 ± 3.2
Boyd Mill Pond	17.7 ± 9.5	18.0 ± 9.4	27.2 ± 13.6	21.1 ± 3.8	47.1 ± 6.0
Lake Greenwood	16.2 ± 6.1	32.1 ± 14.2	16.7 ± 4.8	11.2 ± 2.8	68.3 ± 13.9
Detection limit			0.18 mg/kg dry wt		
Arsenic					
Lake Robinson	0.4 ± 0.0	0.9 ± 0.3	0.4 ± 0.0	0.1 ± 0.0	0.2 ± 0.1
Lake Conestee	5.5 ± 1.0	7.8 ± 1.3	7.6 ± 2.1	0.1 ± 0.0	0.1 ± 0.1
Boyd Mill Pond	0.7 ± 0.4	0.5 ± 0.1	0.8 ± 0.4	0.3 ± 0.1	0.1 ± 0.0
Lake Greenwood	1.1 ± 0.7	0.6 ± 0.3	0.5 ± 0.1	0.2 ± 0.0	0.3 ± 0.0
Detection limit			0.02 mg/kg dry wt		
Selenium					
Lake Robinson	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	1.3 ± 0.1	6.6 ± 0.1
Lake Conestee	1.0 ± 0.1	0.1 ± 0.0	1.2 ± 0.3	0.9 ± 0.0	4.9 ± 0.5
Boyd Mill Pond	0.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.1	1.0 ± 0.1	5.4 ± 1.1
Lake Greenwood	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.9 ± 0.0	4.2 ± 0.2
Detection limit			0.03 mg/kg dry wt		
Silver					
Lake Robinson	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Lake Conestee	1.6 ± 0.1	0.9 ± 0.1	1.2 ± 0.3	0.1 ± 0.0	0.9 ± 0.7
Boyd Mill Pond	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Lake Greenwood	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Detection limit			0.02 mg/kg dry wt		
Cadmium					
Lake Robinson	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.3
Lake Conestee	2.1 ± 1.0	3.2 ± 1.5	3.2 ± 2.4	0.0 ± 0.0	0.7 ± 0.0
Boyd Mill Pond	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.4 ± 0.1
Lake Greenwood	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.5 ± 0.1
Detection limit			0.03 mg/kg dry wt		
Lead					
Lake Robinson	8.6 ± 4.2	3.5 ± 1.2	5.9 ± 1.2	0.1 ± 0.0	1.7 ± 0.1
Lake Conestee	102.9 ± 1.0	81.2 ± 7.5	105.4 ± 6.9	0.1 ± 0.0	1.7 ± 0.1
Boyd Mill Pond	5.9 ± 3.4	2.5 ± 0.9	7.6 ± 4.4	0.1 ± 0.0	1.8 ± 0.2
Lake Greenwood	5.0 ± 2.1	4.8 ± 2.4	5.6 ± 1.6	0.1 ± 0.0	1.7 ± 0.1
Detection limit			0.01 mg/kg dw		

^a All values represent mean ± standard error and are reported as mg/kg dry weight. Concentrations below detection limit are reported at the detection limit.

^b $n = 3$ for samples during an individual season.

^c $n = 7$.

^d $n = 2$.

present in Lake Conestee, and the results of a previous study from Schreiber et al. [3] that indicated significantly elevated levels of the liver enzyme ethoxyresorufin-*O*-deethylase (EROD) in these same fish. Increased EROD levels in previous studies have been shown to correlate to environmental concentrations of PAHs [29,30]. One possible explanation for the lack of correlation between BF values in this study from Lake Conestee and EROD data (from the same fish) and PAHs known to be present in Lake Conestee sediments could be that

PAHs, though present in the sediments, are not bioavailable. Possibly the increased EROD induction observed by Schreiber et al. [3] was attributable to the exposure of largemouth bass to compounds other than PAHs that are known to induce EROD, such as polychlorinated biphenyls, polychlorinated dibenzofurans, or dibenzo-*p*-dioxins [31]. These results are further supported by the lack of glutathione *S*-transferase and uridine-5'-diphospho-glucuronosyltransferase induction observed by Schreiber et al. [3], which are both phase II

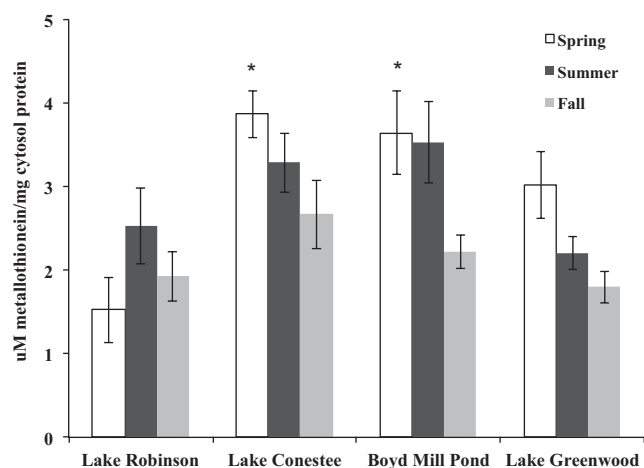


Fig. 4. Concentration of metallothionein in largemouth bass. Significant differences between sites compared with Lake Robinson in the same season are indicated by * ($p < 0.05$, two-way analysis of variance). Data presented as mean \pm standard error mean ($n = 7$).

enzymes that have been shown to increase after exposure to PAHs [31].

Seasonal trends were only observed for four-ring PAHs in the present study. The pattern of observing the highest metabolite concentrations in the coldest seasons is similar to the findings of Gorbi and Regoli [15] and Ruddock et al. [32], who investigated metabolite concentrations in eels (*Anguilla anguilla*). This difference has been attributed to the lower metabolism of fish in colder temperatures, leading to decreased feeding and the concentrating of metabolites in the bile caused by reabsorption of water occurring across the wall of the gallbladder [17].

In addition to its use as a biomarker of PAH exposure, BF analysis can also give insight into the type(s) of PAHs present in a watershed [17]. Polycyclic aromatic hydrocarbon contamination originating from a petrogenic source (i.e., motor oil) shows a dominance of two- and three-ring compounds, and PAHs from a pyrogenic origin (i.e., combusted hydrocarbons) are dominated by four- and five-ring compounds [13,28]. In the present study, a clear dominance of two-ring PAHs was seen compared with four- or five-ring PAHs, indicating the source of PAHs to be from petrogenic origins. This makes sense from a watershed-level perspective, with the input of petrogenic PAHs coming from the City of Greenville, ultimately being deposited into Lake Conestee and transported downstream to Boyd Mill Pond and Lake Greenwood. A common link between PAHs and urbanization is the increased amount of automobile emissions, which would lead to increases in pyrogenic PAHs.

The oil released from the 1996 spill, diesel fuel oil No. 2, typically contains a mixture of C_{10} through C_{19} hydrocarbons, of which approximately 35% are aromatic hydrocarbons [26]. Based on this chemical composition, exposure to fuel oil No. 2 would most likely result in an increased BF signature of two- or four-ring PAH metabolites. This is supported by Kreitsberg et al. [27] when they analyzed heavy oil from a spill in the Baltic Sea near Estonia and found the dominant PAHs present in the oil to be pyrene and fluoranthene, both four-ring PAHs. Results from the present study show no significant increase in four-ring PAH metabolites downstream of the oil spill site compared with upstream sites. If lingering effects of the oil spill were present, metabolite increases in fish from Boyd Mill Pond would have likely been present because this is the first impoundment downstream of the spill site.

In the present study, concentrations of Cr, Ni, Cu, Zn, As, Se, Ag, Cd, and Pb were analyzed in sediments (Table 1) and water (data not shown) and showed that each metal (or metalloids) was predominantly bound to sediments in these impoundments, with concentrations in the parts per million range for sediments and part per billion range (or below the detection limits) for water. Compared with all other sites during all seasons, Lake Conestee sediments had significantly higher sediment metal concentrations. Metal concentrations of Lake Conestee sediments compared with the ecological screening values (<http://www.epa.gov/reg3hwmd/risk/eco/btag/sbv/fwsed/screenbench.htm>) for sediments (Table 2) show that concentrations in Lake Conestee sediments exceeded these values during all seasons for Cd, Cr, Cu, Pb, and Zn, and exceeded screening values in at least one season for Ni and Ag. No site, except for Lake Conestee, exceeded screening values during any season. These results indicate that Lake Conestee sediments are a sink for metals and pose a potential threat to organisms inhabiting the lake. Long et al. [33] developed effects-range thresholds for sediment metal concentrations based on sediment chemical concentrations. These thresholds correspond to sediment concentrations where adverse effects occasionally occur (effects-range-low) or frequently occur (effects-range-high). Sediment concentrations of Cd, Cr, Cu, Pb, Ni, Ag, and Zn in Lake Conestee were above the effects-range-low concentrations but below the effects-range-high concentrations, which indicates that, based solely on chemical concentrations, adverse effects would be expected to occur occasionally because of exposure of these metals. Similar to concentrations of metals observed in water and liver, muscle metal concentrations showed no significant differences from the reference site, except As at Boyd Mill Pond and Lake Greenwood. As is a common component in wood preservatives [34], and the elevated concentrations observed may have been attributable to the high density of boat docks present at these two impounds compared with the reference site. Metallothionein is known to be induced by Cd, Zn, Cu, Pb, and Ag in a variety of organisms, including fish, mammals, insects, and crustaceans [21,31]. In the present study, significant differences in MT concentrations attributable to both site and season were found. The elevated MT levels observed in Lake Conestee fish correspond with elevated concentrations of metals known to induce in MT. However, Boyd Mill Pond also showed elevated MT concentrations compared with the reference site, which was unexpected, given that no metal concentrations at Boyd Mill Pond differed from control concentrations. Possibly this elevation was attributable to factors other than metal exposure known to induce MT (e.g., increased glucocorticoid and peptide hormones or estrogenic polychlorinated biphenyls)

Table 2. U.S. Environmental Protection Agency screening level for sediment metals

Metal	Freshwater ^a sediment screening benchmarks
Chromium	43.4
Nickel	22.7
Copper	31.6
Zinc	121
Arsenic	9.8
Selenium	2
Silver	1
Cadmium	0.99
Lead	35.8

^a All concentrations in mg/kg. Data available at <http://www.epa.gov/reg3hwmd/risk/eco/btag/sbv/fwsed/screenbench.htm>.

[31]. Further investigation into elevate MT concentrations at Boyd Mill Pond are warranted. The significant effect based on season was also observed with respect to MT concentrations, with spring and summer concentrations being significantly greater than those in the fall. The effect of season on liver MT concentrations in fish has not been widely studied. The results of the present study were similar to those observed by Barhouni et al. [35] that found the highest concentrations of MT in the livers of *Salaria basilisca* at a contaminated field site occurred during the summer and the lowest concentrations during the winter. The seasonal effect observed in the present study may have been partially attributable to overall fish metabolism being greater during seasons with warmer water temperatures or possibly linked to the reproductive cycle of largemouth bass; however, this is speculation, and further studies are necessary to understand the mechanism(s) driving the seasonal changes observed in MT.

The MT results of the present study are in contrast to the finding of Schreiber et al. [3], who concluded that Pb was not bioavailable to largemouth bass (same fish as the present study) based on the biomarker δ -aminolevulinic acid dehydratase tested/measured in blood samples. Though this difference may be an artifact of the specificity of δ -aminolevulinic acid dehydratase as a biomarker for lead, MT is also known to be induced by Pb [21,31].

The data collected in the present study, in combination with previous work in this same watershed—specifically Schreiber et al. [3]—highlight the complex nature of ecotoxicology and the continued need for a multiple lines of evidence approach in field-based studies. In this case, when all available evidence (both chemical and biological) is considered, no definitive conclusion can be drawn with regard to PAH or metal bioavailability in the Reedy River watershed because of contrasting biomarker results (PAH: EROD and BF; metals: δ -aminolevulinic acid dehydratase and MT). Ultimately, even as frustrating as it may be, the present study combined with Schreiber et al. [3] demonstrates the strength of a multiple lines of evidence approach by not drawing overall conclusions based on a single line of evidence. Watershed level assessments are difficult because of spatial variability and connectedness of sites within the same watershed. The impacts that occur at any single site have the potential to affect all sites downstream of its location. Because of this complexity, the use of integrated chemical and biological analysis is valuable because it allows for a multiple lines of evidence approach to assessment.

CONCLUSION

In the present study, the impacts of PAHs and metals were investigated using chemical and biological indicators to determine the relationship between these two measurement tools at the watershed-scale. Chemical analyses indicated significantly elevated concentrations of PAHs and metals at Lake Conestee, the first impoundment downstream of the urbanized City of Greenville. Bile fluorescence analyses suggest that the PAHs in Lake Conestee are not readily bioavailable to largemouth bass, and that the likely sources of PAHs in this watershed are of a petrogenic origin. Metallothionein concentrations indicate that the metals present in Lake Conestee are bioavailable and are subject to seasonal variability. No chemical or biological indicators show evidence for lingering effects from the large oil spill that occurred within this watershed approximately 10 years before the present study. Overall, the present study combined with data from Schreiber et al. [3] highlights the

importance of a multiple lines of evidence approach to watershed assessments.

SUPPLEMENTAL DATA

Table S1. (49 KB XLSX)

Acknowledgement—The present study was supported by grants from the South Carolina Water Resources Center, the Saluda-Reedy Watershed Consortium, and the V.K. Rasmussen Foundation. The present study was conducted with the approval of the Clemson University Animal Research Committee, under the Office of Research Compliance and is in accordance with national and Clemson University guidelines for the protection of human subjects and animal welfare.

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