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Relating fish health and reproductive metrics to contaminant bioaccumulation at the Tennessee Valley Authority Kingston coal ash spill site

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Abstract A 4.1 million m^3 coal ash release into the Emory and Clinch rivers in December 2008 at the Tennessee Valley Authority's Kingston Fossil Plant in east Tennessee, USA, prompted a long-term, large-scale biological monitoring effort to determine if there are chronic effects of this spill on resident biota. Because of the magnitude of the ash spill and the potential for exposure to coal ash-associated contaminants [e.g., selenium (Se), arsenic (As), and mercury (Hg)] which are bioaccumulative and may present human and ecological risks, an integrative, bioindicator approach was used. Three species of fish were monitored—bluegill (*Lepomis macrochirus*), redear sunfish (*L. microlophus*), and largemouth bass (*Micropterus salmoides*)—at ash-affected and reference sites annually for 5 years following the spill. On the same individual fish, contaminant burdens were measured in various tissues, blood chemistry parameters as metrics of fish health, and various condition and reproduction indices. A multivariate statistical approach was then used to evaluate relationships between contaminant bioaccumulation and fish metrics to assess the chronic, sub-lethal effects of exposure to the complex mixture of coal ash-associated contaminants at and around the ash spill site. This study suggests that while fish tissue concentrations of some ash-associated contaminants are elevated at the spill site, there was no consistent evidence of compromised fish health linked with the spill. Further, although relationships between elevated fillet burdens of ash-associated contaminants and some fish metrics were found, these relationships were not indicative of exposure to coal ash or spill sites. The present study adds to the weight of evidence from prior studies suggesting that fish populations have not incurred significant biological effects from spilled ash at this site: findings that are relevant to the current national discussions on the safe disposal of coal ash waste.

Keywords Coal-ash · Fish · Fish health · Bioaccumulation · Contaminant · Arsenic · Mercury · Selenium

Introduction

Creating strong linkages between environmental pollution exposure and health effects in wild animal populations can be difficult (Rose 2000). When there is large release of a contaminant and conspicuous death of a large number of organisms these linkages are fairly straightforward. However, in the bulk of contaminated ecosystems linking environmental perturbations and sub-lethal population impacts often requires teasing apart multiple conflating factors. For example, creating links between a released contaminant and an animal population may require a combination of knowledge of the environmental history of the contaminated area, the environmental history of surviving organisms, controlled exposures of cell cultures or whole animals at one or multiple life stages to the released

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contaminant, and modelling approaches that extrapolate sub-organismal and individual effects to a population (Rose 2000). Unfortunately, controlled experimental studies aimed at mechanistically cataloguing sub-lethal effects and scaling to see if these effects have population impacts can take several years to decades to complete. As a result, many of the initial conclusions on the sub-lethal effects of environmental contamination are based on relational data from uncontrolled field and laboratory studies, although these initial studies can be important for generating hypotheses for further mechanistically-focused experimentation. Perhaps more importantly, management and restoration decisions must be made on a much shorter timescale than that allowed by controlled experimentation. Frequently, relational studies represent the best information available on a particular ecosystem for forming urgent policy decisions.

The effects of coal ash, the byproduct of coal combustion, on aquatic biota have become a major concern to environmental regulators. Depending on the source of the coal used as fuel, coal ash can contain high concentrations of contaminants such as arsenic (As), mercury (Hg), and selenium (Se), which can be toxic to biota and can bioaccumulate. These contaminants can then be transferred to humans consuming fish and other aquatic organisms, thereby creating possible public health concerns. Because the chemical make-up of coal ash varies by coal source, reported effects of coal ash on biota can be varied ranging from near complete reproductive failure (Lemly 2002) to negligible and undetectable effects (Souza et al. 2013). Although the exact mechanisms and toxic constituents of coal ash are not known, coal ash spills have been linked to reduced nest success in birds (King et al. 1994) and skeletal deformities in fish (Lemly 2002).

Concerns about the effects of coal ash on aquatic organisms were again brought to the fore following the rupture of a retention pond dike in 2008 at the Tennessee Valley Authority's Kingston Fossil Plant (TVA KIF). This breach spilled coal ash into the Emory River, in east Tennessee, USA resulting in the largest disaster of its kind in US history. While acute effects of the TVA KIF coal ash spill to aquatic animals were conspicuous, including the killing of an unknown number of fish, mussels, benthic macroinvertebrates, and other aquatic organisms (Lemly and Skorupa 2013; Bryan et al. 2012; Otter et al. 2013; Souza et al. 2013), ongoing monitoring of the spill site has not suggested any major threats to humans, fish or wildlife since the initial incident: drinking water has remained safe and water quality has generally not exceeded regulatory criteria.

In the present study, the effects of coal ash contamination on biota were further explored through relating bioindicators of fish from three reference sites and three TVA KIF ash-affected sites to assess the fish response to coal ash. This approach involved relating a suite of selected biological responses including biochemical markers, condition indices and reproductive health markers (Adams and Greeley 2000) to As, Hg, and Se burdens in fishes.

Since coal ash contains a number of potentially toxic contaminants and exposure to coal ash contaminants at the KIF site has changed over time due to remediation efforts and riverine hydrologic processes, the response of fish to ash-associated contaminants is likely complex. Statistically relating fish metrics with bioaccumulation results from the KIF site can be a first step towards understanding the response to coal ash exposure over time. To this end, the TVA KIF coal ash fish health, reproduction and bioaccumulation monitoring data were used to determine if there were relationships between contaminants in fish fillets and fish metrics and, if these relationships exist, whether they were related to site and ash exposure. This study uses a statistical approach to highlight links between fish health, reproduction and selected coal ash-related contaminant burdens. This study can then serve as an essential first step for generating hypotheses and associations that can be further explored in the future using carefully designed studies targeting a mechanistic understanding of the effects of coal ash on aquatic animal health.

Materials and methods

Study area

The TVA KIF is located adjacent to the confluence of the Emory and Clinch rivers in east Tennessee, USA (Fig. 1). Approximately 90 % of the ash spilled into the Emory River, some of which was pushed up to Emory River mile 6 (Tennessee Valley Authority (TVA) 2009). The remaining ~10 % of the ash spilled into an embayment to the north of the TVA KIF, and downstream into the Clinch River (Tennessee Valley Authority (TVA) 2009). Following the spill, fish were collected each spring for this study from 2009 to 2013 at three ash-affected sites—Emory River mile 3.0 (spill site; hereafter, S1), Emory River mile 0.9 (approx. 2 mi or 3 km downstream of the spill site; hereafter S2) and Clinch River mile 1.5 (approx. 6 mi or 10 km downstream of the spill site; hereafter S3)—and three reference sites that were unaffected by the spill—Emory River mile 8.0 (approx. 5 mi or 8 km upstream of the spill site; hereafter R1), Little Emory River mile 2.0 (approx. 2 mi or 3 km upstream of the confluence of the Emory and Little Emory rivers; hereafter R2), and Clinch River mile 8.0 (approx. 4 miles or 6 km upstream of the confluence of the Clinch and Emory rivers; hereafter R3). Ash was dredged from the ash-affected Emory River sites (S1, S2)

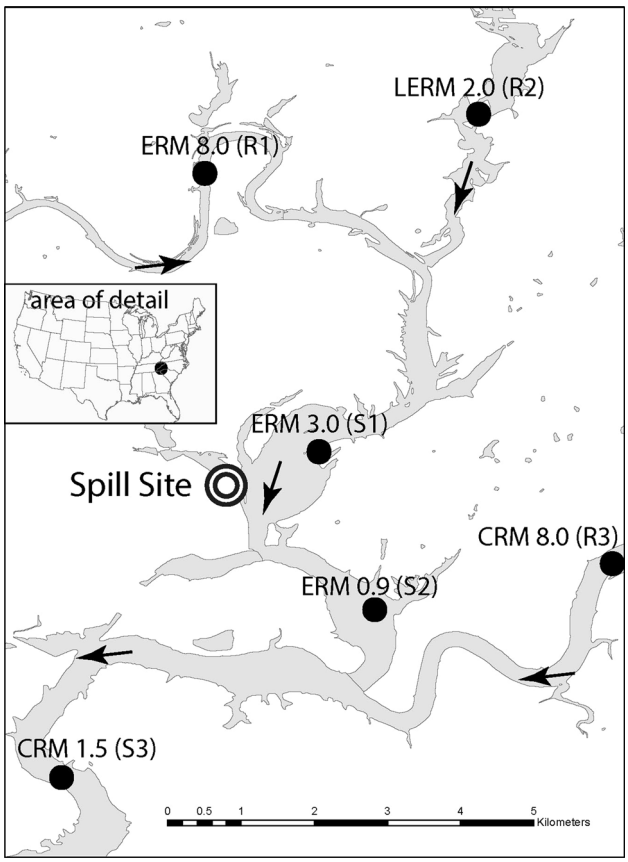

Fig. 1 Map of study area showing locations of ash-affected (S) and reference (R; not affected by ash) monitoring sites where fish were collected including ash-affected sites Emory River mile 3.0 (ERM 3.0, S1), Emory River mile 0.9 (ERM 0.9, S2), and Clinch River mile 1.5 (CRM 1.5, S3) and reference sites Emory River mile 8.0 (ERM 8.0, R1), Little Emory River mile 2.0 (LERM 2.0, R2), and Clinch River mile 8.0 (CRM 8.0, R3). *Arrows* indicate the direction of flow

from 2009 to 2010 where 65 % of the 4.1 million m^3 of spilled coal ash were removed (Bartov et al. 2012). Site R1 was used in this study as a reference site; however, it has received considerable environmental contamination from a now-closed, upstream paper mill (Bartov et al. 2012). Site R3 has also received considerable environmental contamination from an upstream Department of Energy facility for many years (Bartov et al. 2012). While the reference sites chosen for this study are far from pristine, all regionally proximate, and therefore, relevant, candidate reference sites have been impacted by some form of legacy alteration or contamination. This region is one of high freshwater biodiversity and geologic heterogeneity (Pracheil et al. 2014) thereby underscoring the importance of choosing spatially proximate reference sites.

Pore-water leached from spill site sediments contained elevated levels of As, Se, Hg, boron, strontium, barium, uranium, chromium, iron, and manganese (but not lead) compared with non-impacted upstream sites (Ruhl et al. 2009, 2010; Bartov et al. 2012; Deonarine et al. 2013). Concentrations of many elements were measured in these fishes (e.g., aluminum, antimony, As, barium, beryllium, boron, cadmium, calcium, chromium, cobalt, copper, iron, lead, magnesium, manganese, Hg, molybdenum, nickel, potassium, Se, sodium, strontium, thallium, vanadium, zinc). However, preliminary correlations with this suite of elements showed only consistent significant trends with As, Hg, and Se, so the present study focuses on these contaminants. Also, the focus was placed on As, Hg, and Se because of their relative abundance in fish fillets analyzed (other contaminants had very few measurements above the LOD) and extensive documentation in the literature of these three contaminants producing biological effects on fishes and other aquatic organisms. A Hg source-apportionment study using stable isotopes conducted after dredging was completed could not conclusively determine whether Hg from sediment in ash-affected areas was a result of legacy contamination or the coal ash spill, although it is certain that at least some of the Hg was sourced by the coal ash spill (Bartov et al. 2012).

Target fish species

To assess impacts on aquatic biota, bioaccumulation and a variety of fish health metrics were monitored in bluegill (*Lepomis macrochirus*), redear sunfish (*L. microlophus*), and largemouth bass (*Micropterus salmoides*). These species were selected because they are abundant in the Emory and Clinch rivers and are commonly caught and consumed by local anglers. Furthermore, these species are relatively short-lived and generally have a limited home range (Etnier and Starnes 1994), so fillet contaminant concentrations should be representative of exposure at the site of collection (Peterson et al. 1996). These fish species also represent a variety of trophic levels: bluegill and redear sunfish feed on a varied diet of insects, crustaceans, and other zoobenthos (Etnier and Starnes 1994), but redear sunfish in this system have a preference for mollusks (Otter et al. 2013). Largemouth bass, on the other hand, are top predators eating fishes such as bluegill (Etnier and Starnes 1994). The effects of contaminants on the health of fish of different trophic levels were examined because some contaminants, such as As, Hg and Se, have been shown to biomagnify with increasing trophic position (Barwick and Maher 2003). That is, the higher the trophic level of the fish, the higher the contaminant concentration. For spatial and temporal comparability and to minimize effects of covariance between size and contaminant concentrations, fish only of sizes large enough to be caught by anglers (generally 50–150 g for sunfish, and 500–2500 g for largemouth bass, total weight) were collected for bioaccumulation and fish health studies. Fish were collected

using a boat electrofisher: bluegill and largemouth bass during April–June of 2009–2013 and redear sunfish during April–June 2010–2013. Numbers of fish per year for each site and species combination generally ranged from 10 to 20.

Sample processing and calculation of health and condition metrics

Up to 1 mL of blood was collected from each fish while still in the field. Upon return to the laboratory, all fish were euthanized with MS-222. Fish were then dissected and major organs (liver, kidneys and ovaries) removed prior to weighing. Metrics of fish health including measures of bioenergetics, hematology and immune function, carbohydrate-protein metabolism, electrolyte homeostasis, liver condition, and overall fish condition, were assessed for each fish. Several metrics of fish condition (CI) were assessed including the liver-somatic, visceral-somatic, and spleno-somatic indices and were calculated as

$$\mathrm{CI} = \frac{M_o}{M_b} \times 100$$

where M_o = wet organ mass (g) and M_b = wet body mass (g). Overall condition factor C_f was calculated as

$$C_f = \frac{M_T}{L_T^3} \times 100$$

where M_T = total wet mass (g) and L_T = total length (cm).

Blood chemistry analyses

Blood hematocrit and leucocrit were determined by the standard capillary tube and centrifugation method. Fourteen blood chemistry metrics were analyzed with an Abaxis VetScan II (Abaxis, Union City, CA, USA) clinical analyzer and tested for several analytes that are known as indicators of physiological response in fish (Abaxis test rotor #500-0038). Analysis of all 14 blood chemistry metrics (Table 1) required 100 µL of blood from each fish.

Reproductive condition assessment

Representative pieces of ovarian tissue were placed in vials containing a half-strength solution of Karnovsky's Fixative for later analysis of ovary stage, oocyte (immature developing eggs) condition, and fecundity. Fish reproductive condition was quantified by sizing, staging and counting all oocytes above size thresholds for active yolk accumulation (vitellogenesis) that were contained in a weighed subsample of ovary. From these measurements, batch fecundity, numbers of vitellogenic oocytes and numbers of atretic oocytes were estimated for each ovary.

Because the fish species examined had differing life history strategies, accommodating species-specific patterns of oocyte development was a requirement for estimating fecundity. Each species can spawn multiple times during the breeding season; therefore, fecundity estimates considered only the most mature clutch of developing oocytes in pre-spawn fish, or post-ovulatory follicles in immediately post-spawn fish, following methods outlined in Greeley et al. (2012). Batch fecundities and the abundance of vitellenogenic and atretic oocytes were estimated as

$$N = \frac{N_e}{M_s} M_o$$

where N_e is the number of oocytes or post-ovulatory follicles in a clutch, the total number of vitellogenic oocytes, or the abundance of atretic oocytes in the analyzed ovary piece, M_s is the mass of the ovarian subsample, and M_o is the mass of the entire ovary.

Bioaccumulation analyses

Fish samples were shipped frozen (<−10 °C) to Pace Analytical Services, Inc. (Green Bay, WI) for homogenization, moisture determination, and analysis of As, Hg, and Se. For As and Se quantification, tissue aliquots were weighed (wet) and digested in nitric acid (EPA method SW846-3050) prior to analysis using ICP-MS (EPA method SW846-6020). Tissue samples were analyzed for Hg directly (EPA method SW846-7473) using a Direct Mercury Analyzer (Milestone, Sorisole, Italy). All quality assurance procedures (e.g., blanks, matrix spikes) were conducted as specified in the analytical method.

Method detection limits (MDL) for this project were calculated based on historical blank concentrations as described by the following equation:

$$\mathit{Project\ MDL} = \mathit{avg} + 3\sigma$$

where avg is the mean of historical method blank concentrations and σ = SD of the population of historical method blank concentrations. The MDL (prior to sample-specific adjustment to account for actual weight of the digested aliquot) was 0.0142 mg/kg for As, 0.0753 mg/kg for Se and 0.001 mg/kg for Hg. Standard reference materials (SRM) included lobster hepatopancreas (TORT-2) and dogfish liver tissue (DOLT-4) from the National Research Council in Canada (NRC) and Lake Michigan fish tissue from the National Institute of Standards and Technology (NIST-1947). The TORT-2 SRM was digested and analyzed with each batch of samples, while the DOLT-4 and NIST-1947 SRMs were alternated with every other sample batch. Acceptance criteria for Se recovery in TORT-2 were 80–120 % of the certified value of 5.63 mg/kg. In addition, chicken fillets were used as the matrix for the laboratory

Table 1 Description of the physiologic relevance of bioindicator metrics (abbreviation) of fish health and condition by functional response group

Functional response group	Bioindicator	Physiologic indication
Bioenergetics	Amylase (AMY)	Converts starch into sugars
Organ Function	Alanine transferase (ALT)	Liver function
	Blood urea nitrogen (BUN)	Kidney and gill function
	Creatinine (CREAT)	Kidney function
	Total bilirubin (TBIL)	Liver function
	Alkaline phosphatase (ALP)	Bone formation
Carbohydrate-protein metabolism	Glucose (GLU)	Metabolic efficiency
	Blood protein (BPRO)	Liver and general inflammation
	Globulin (GLOB)	Liver and kidney function
	Albumin (ALB)	Liver and kidney function
	Phosphorus (PHOS)	Indicator of kidney, liver, bone disease
Electrolyte homeostasis	Calcium (Ca), Sodium (Na)	Function of most organs including liver and kidney
Fish Condition	Condition factor (C_f)	Index of plumpness
	Gonadosomatic index (GSI)	Index of reproductive potential
	Hepatosomatic index (LSI)	Index of energy reserves
	Spleen somatic index (SSI)	Index of immune response
	Visceral somatic index (VSI)	Index of overall condition
Reproductive health	Vitellogenic oocytes (VO)	Number of oocytes with yolk
	Atresia (ATR)	Number of atretic oocytes
	Batch fecundity (FEC)	Number of eggs produced per clutch

control spike with each batch of samples, with acceptance criteria of 80–120 % of the spike. All samples were analyzed and reported on a wet-weight basis. Values below detection limits were excluded from analyses. Frequencies of fillet concentrations below detection limits are shown in Appendix A (This and all appendices are provided in online Supplemental Material).

Data analysis

Bioaccumulation values below the MDL were censored from all analyses. Although fillet, ovary, and liver bioaccumulation data were collected, analyses used data from fillets because that was the only appropriately-large dataset. The assumption of focusing on fillet bioaccumulation data was that it was related to concentrations found in other organs. This assumption was validated using Pearson's correlations between liver and fillet and ovary and fillet concentrations to understand how well or whether fillet bioaccumulation was related to bioaccumulation in other tissue types (Appendix B in online Supplemental Material).

Fish health, reproduction, and bioaccumulation data were examined for normality by comparing the median and mean of the data set (they were considered approximately normal if they differed by a factor of <3), and visually inspecting a normal-quantile plot of the data to see if it differed substantially from a 1:1 line. Data that were not determined to be normal were $\log_{10}$ transformed and again examined for normality using the above methods. Just number of atretic oocytes—a reproductive metric—was found to violate normality assumptions and was log-transformed.

Multivariate analyses of covariance (MANCOVA; proc mixed, SAS) were used for each species to detect differences among contaminants and fish length, site, and year of collection. Upon conducting routine examinations of the data (e.g., Pearson's correlations), it was discovered that As, Hg, and Se are correlated with each other. Due to the non-independence of these data and influence of covariates, a MANCOVA was chosen to test for differences for each species. The MANCOVA was also used for each fish species to determine relationships between 1. blood chemistry metrics and contaminants, 2. fish condition metrics and contaminants, 3. reproductive health metrics and contaminants where length was used as a covariate in each test. Post-hoc Tukey's honestly significant difference tests were used to evaluate differences between sites for all MANCOVAs.

Because there are so many possible site, year and species combinations for each measured variable (6 sites $\times$ 5 years $\times$ 3 species $=$ 90 possible combinations), individual pairwise differences were not reported although

specific comparisons between sites were highlighted that help to illustrate conclusions. Instead, for measures of fish health (e.g., blood chemistry, fish condition, reproduction) the critical, and consistent comparisons made across all sites, were that made between reference sites and spill sites. These findings were presented as a summary of least-squares (LS) means for each site type with associated p-values for the difference between LS means.

Results

Bioaccumulation

Across all sites, bluegill had the highest Se concentration in 2010 whereas redear sunfish had the highest Se concentrations in 2011 (Fig. 2; Appendix C in online Supplemental Material). Across all species, spill sites generally had higher Se burdens than reference sites. Fillet burdens of Se were highest in 2010 or 2011 depending on the site by species combination. Selenium concentrations in largemouth bass did not show consistent patterns with respect to site. For Hg and As, the trends were quite different. Reference sites, particularly R1 and R3 had the highest Hg values in nearly all sample periods for all species except largemouth bass.

While Hg levels rose at all sites and species through the study period, the most dramatic increases were found in largemouth bass at site R1. For largemouth bass, a post hoc Tukey's test showed that Hg concentrations from site R1 were significantly different those from all other sites and there were no significant differences between any other sites. Post-hoc Tukey's tests also showed there were elevated concentrations of As in bluegill from spill sites in 2010 and in largemouth bass from spill sites from 2010 to 2011—the first years following the spill.

Nearly all main-effects and their interactions were significant in the MANCOVA examining the effect of site type (reference or ash-affected; Table 2). Year was only significant for redear sunfish, although nearly so for bluegill. Tukey's tests showed that Se was significantly higher in ash-affected sites for all three species, and largemouth bass had significantly higher Hg at reference sites than at spill sites (Table 3).

Bioaccumulation and blood chemistry functional response groups

There were no consistent patterns in blood chemistry functional response groups over years for any species (Appendix D in online Supplemental Material). While all overall MANCOVA models for blood chemistry were significant for all species, no main-effects were significant for any species (Table 4). However, the interaction of main-effects were significant for largemouth bass, but not for other species. Posthoc Tukey's tests showed no

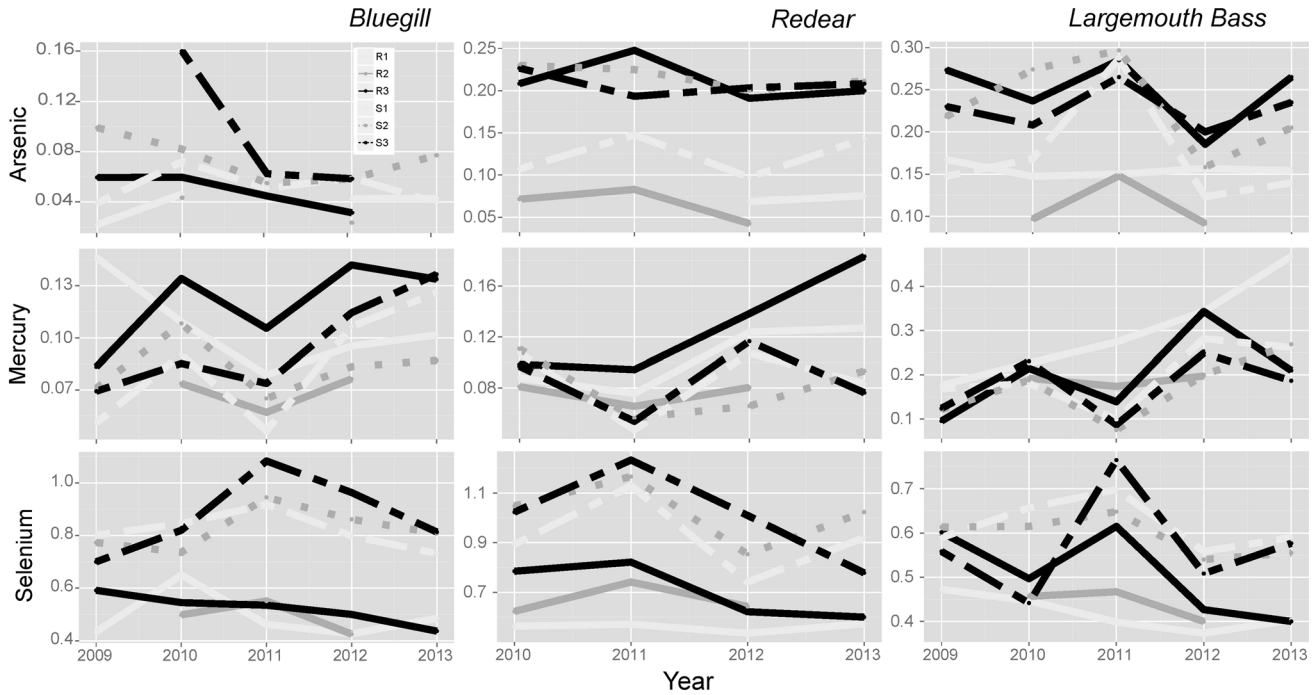

Fig. 2 Time-series of mean arsenic, mercury and selenium fillet concentrations (mg/kg wet-weight) by site and species. *Error bars* are omitted for clarity. Sites abbreviations are as shown in Fig. 1

Table 2 Results for MANCOVAs examining the response of metal and metalloid contaminant (in this table, hereafter, metal) concentrations to effects of collection site (hereafter, spill, denoting reference or ash-affected site), year, metal × spill, metal × year, year × spill, and metal × site × year

Species	Effect	Num df	Denom df	F	P
Bluegill	Metal	**2**	**421**	**2369.08**	**<0.0001**
	Spill	**1**	**421**	**185.95**	**<0.0001**
	Year	4	421	2.39	0.0503
	Metal × spill	**2**	**421**	**198.39**	**<0.0001**
	Metal × year	**8**	**421**	**4.96**	**<0.0001**
	Year × spill	**4**	**421**	**3.33**	**0.0106**
	Metal × spill × year	**8**	**421**	**3.53**	**0.0006**
Redear	Metal	**2**	**379**	**1695.48**	**<0.0001**
	Spill	**1**	**379**	**121.31**	**<0.0001**
	Year	**3**	**379**	**7.28**	**<0.0001**
	Metal × Spill	**2**	**379**	**105.25**	**<0.0001**
	Metal × Year	**6**	**379**	**10.33**	**<0.0001**
	Year × Spill	3	379	1.27	0.2860
	Metal × spill × year	**6**	**379**	**2.58**	**0.0184**
Lg.mouth bass	Metal	**2**	**484**	**521.56**	**<0.0001**
	Spill	**1**	**484**	**16.27**	**<0.0001**
	Year	4	484	1.48	0.2072
	Metal × spill	**2**	**484**	**37.10**	**<0.0001**
	Metal × year	**8**	**484**	**12.02**	**<0.0001**
	Year × spill	4	484	0.34	0.4417
	Metal × spill × year	**8**	**484**	**2.41**	**0.0148**

Overall model results are shown in the row with effect "metal" and all results are given as numerator degrees of freedom, denominator degrees of freedom, F-value. and P value. Significant P values ($\alpha = 0.05$) for each covariate from type III sum-of-squares are shown in bold

Table 3 Results of Tukey's post hoc tests (completed following the MANCOVA shown in Table 2) comparing metal and metalloid concentrations of least square means (LS means and the SE of the LS means) at reference and spill sites (P value Diff LS Means)

Species	Metal/metalloid	Site type	LS means	SE	P value (diff LS means)
Bluegill	As	Reference	0.0426	0.0132	0.7671
		Spill	0.0654	0.0108	
	Hg	Reference	0.1036	0.0100	0.9032
		Spill	0.0895	0.0091	
	Se	Reference	**0.4998**	**0.0100**	**<0.0001**
		Spill	**0.8407**	**0.0091**	
Redear	As	Reference	0.1341	0.0151	0.1460
		Spill	0.1832	0.0133	
	Hg	Reference	0.1091	0.0138	0.7913
		Spill	0.0844	0.0133	
	Se	Reference	**0.6375**	**0.0138**	**<0.0001**
		Spill	**0.9842**	**0.0132**	
Large mouth bass	As	Reference	0.1785	0.0127	0.3936
		Spill	0.2110	0.0112	
	Hg	Reference	**0.2382**	**0.0116**	**0.0059**
		Spill	**0.1811**	**0.0112**	
	Se	Reference	**0.4548**	**0.0116**	**<0.0001**
		Spill	**0.5943**	**0.0112**	

Significant P values ($\alpha = 0.05$) are shown in bold

Table 4 Results for MANCOVAs examining the response of blood chemistry concentrations to effects of functional response group (hereafter, function: bioenergetics, carbohydrate–protein metabolism, electrolyte balance, hematology, organ function,), arsenic (As), mercury (Hg), selenium (Se), fish length (Length), spill (reference or ash-affected sites), year, function × spill, function × year, year × spill, and function × site × year

Species	Effect	Num df	Denom df	F	P
Bluegill	Function	**4**	**1585**	**80.97**	**<0.0001**
	Spill	1	1585	0.14	0.7118
	Year	4	1585	2.01	0.0913
	Arsenic	1	1585	0.43	0.5134
	Mercury	1	1585	0.42	0.5193
	Selenium	1	1585	0.14	0.7067
	Length	1	1585	0.33	0.5662
	Function × spill	4	1585	0.10	0.9828
	Function × year	16	1585	0.56	0.9137
	Year × spill	4	1585	0.14	0.9666
	Function × spill × year	16	1585	0.15	1.0000
Redear	Function	**4**	**1658**	**124.32**	**<0.0001**
	Spill	1	1658	0.06	0.8046
	Year	3	1658	2.00	0.3884
	Arsenic	1	1658	0.74	0.9512
	Mercury	1	1658	0.00	0.6332
	Selenium	1	1658	0.23	0.6388
	Length	1	1658	0.22	0.1119
	Function × spill	4	1658	0.29	0.8836
	Function × year	12	1658	1.26	0.2348
	Year × spill	3	1658	0.74	0.5288
	Function × spill × year	12	1658	0.46	0.9395
Lg.mouth bass	Function	**4**	**2142**	**645.77**	**<0.0001**
	Spill	1	2142	0.22	0.6371
	Year	4	2142	2.23	0.0636
	Arsenic	1	2142	0.28	0.5994
	Mercury	1	2142	0.07	0.7913
	Selenium	1	2142	0.45	0.5024
	Length	1	2142	0.32	0.5702
	Function × spill	4	2142	0.51	0.7282
	Function × year	**16**	**2142**	**1.66**	**0.0477**
	Year × spill	**4**	**2142**	**4.25**	**0.0020**
	Function × spill × year	**16**	**2142**	**2.31**	**0.0022**

Overall model results are shown in the row with effect "function" and all results are given as numerator degrees of freedom, denominator degrees of freedom, F-value. and P value. Significant P values ($\alpha = 0.05$) for each covariate from type III sum-of-squares are shown in bold

differences in functional response groups between reference and spill sites (Appendix E in online Supplemental Material).

Bioaccumulation and fish condition metrics

There did not appear to be distinct spatial or temporal trends in condition metric time-series data (Fig. 3). Overall MANCOVA models were significant for all three fish species (Table 5). The main-effect of year as well as the interaction between condition factor type and year was significant for bluegill and redear sunfish. No main-effects were significant in the largemouth bass MANCOVA by themselves, although the interactions between year and site type and condition factor type, year and site type were significant (Table 5). Post-hoc Tukey's tests indicated that redear C_f was higher in reference sites (LS $Means_{ref} = 1.638$, LS $Means_{spill} = 1.594$; P-val: 0.045; Appendix F in online Supplemental Material), but no other comparisons between reference and spill sites were significant.

Bioaccumulation and reproductive health metrics

Both redear and bluegill sunfish had decreases in atretic oocytes from 2010 to 2011 at ash-affected sites (Fig. 4). In these fish, the highest mean atretic oocytes were found at spill

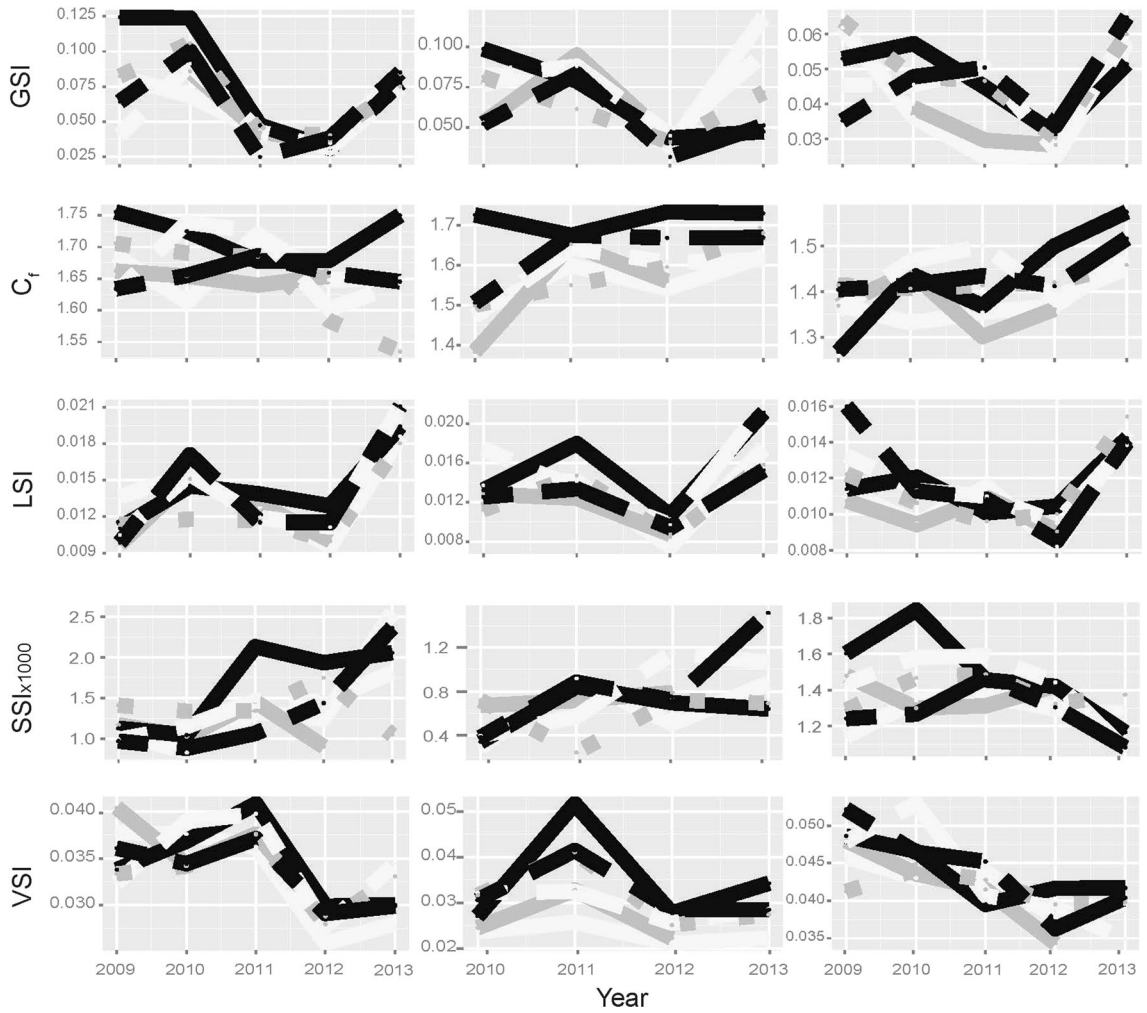

Fig. 3 Time-series of gonodosomatic index (GSI), condition factor (C_f), hepatosomatic index (LSI), splenosomatic index (SSI), viscerosomatic index (VSI). *Error bars* are omitted for clarity. Legend is as in Fig. 2 and sites are as in Fig. 1

sites in the years following the spill (2010 being the first year data were collected following the spill for redear sunfish) and during/immediately after dredging, although site S1 also had the highest fecundity and the largest number of vitellogenic oocytes reported. Redear sunfish showed some additional spatial and temporal trends in reproductive metrics by site and year. While overall MANCOVA models and main-effects of length were significant for all species, the main-effect of Se was significant for both bluegill and redear as well as the main-effect of As for redear (Table 6). Interaction effects of year and site type were additionally significant for bluegill and largemouth bass and the three-way interaction of metric type, year and site type for bluegill. No post hoc Tukey's tests for differences in reproductive metrics between reference and ash-affected sites were significant at the $\alpha = 0.05$ level, although there was a significant difference between reference and ash-affected sites in Vitellogenic oocytes of redear sunfish at the $\alpha = 0.10$ level (LS $\text{Means}_{\text{ref}} = 17{,}694$, LS $\text{Means}_{\text{spill}} = 14{,}740$; P value $= 0.0694$; Appendix G in online Supplemental Material).

Discussion

This is the first study to explicitly relate fish bioaccumulation to biological endpoints at the TVA KIF spill site, relating contaminant bioaccumulation data and a variety of fish biological endpoints. Most importantly, this is the first study from the TVA KIF that draws quantitative links between contaminants and reproductive endpoints: an essential component for scaling biological effects to the population level. Even after looking at these data using multiple approaches both in this study and in prior studies, the weight of findings suggests there is little evidence of major long-term fish health effects of the TVA KIF coal ash spill. For example, Bevelhimer et al. (2014) did not find

Table 5 Results for MANCOVAs examining the response of fish condition values to effects of fish condition (hereafter, condition: condition factor, gonado-somatic index, hepato-somatic index, spleno-somatic index, viscero-somatic index), arsenic (As), mercury (Hg), selenium (Se), fish length (Length), spill (reference or ash-affected site type), year, condition × spill, condition × year, year × spill, and condition × site × year

Species	Effect	Num df	Denom df	F	P
Bluegill	Condition	**4**	**551**	**16,815.9**	**<0.0001**
	Spill	1	**551**	0.02	0.8784
	Year	**4**	**551**	**2.56**	**0.0375**
	Arsenic	1	**551**	0.13	0.7179
	Mercury	1	**551**	0.18	0.6689
	Selenium	1	**551**	1.87	0.1716
	Length	1	**551**	0.56	0.4532
	Condition × spill	4	**551**	0.92	0.4504
	Condition × year	**16**	**551**	**1.83**	**0.0244**
	Year × spill	4	**551**	1.28	0.2750
	Condition × spill × year	16	**551**	0.59	0.8948
Redear	Condition	**4**	**581**	**13,666.0**	**<0.0001**
	Spill	**1**	**581**	**4.68**	**0.0309**
	Year	**3**	**581**	**6.84**	**0.0002**
	Arsenic	1	**581**	1.93	0.1651
	Mercury	1	**581**	0.62	0.4325
	Selenium	1	**581**	2.27	0.1321
	Length	1	**581**	0.23	0.6289
	Condition × spill	4	**581**	1.51	0.1965
	Condition × year	**12**	**581**	**7.30**	**<0.0001**
	Year × spill	3	**581**	0.98	0.4038
	Condition × spill × year	12	**581**	0.51	0.9066
Lg.mouth bass	Condition	**4**	**775**	**5540.29**	**<0.0001**
	Spill	1	**775**	1.19	0.2755
	Year	4	**775**	1.61	0.1701
	Arsenic	1	**775**	0.26	0.6133
	Mercury	1	**775**	0.60	0.4380
	Selenium	1	**775**	0.21	0.6433
	Length	1	**775**	2.89	0.0893
	Condition × spill	4	**775**	1.95	0.0996
	Condition × year	16	775	1.42	0.1251
	Year × spill	**4**	**775**	**2.82**	**0.0241**
	Condition × spill × year	**16**	**775**	**2.17**	**0.0049**

Overall model results are shown in the row with effect "condition" and all results are given as numerator degrees of freedom, denominator degrees of freedom, F-value and P value. Significant P values ($\alpha = 0.05$) for each covariate from type III sum-of-squares are shown in bold

associations between fish blood chemistry metrics and sites of collection beyond the first years after the spill, finding that variation among years was greater than variation among sites. In most cases, while site was often a significant effect in MANCOVAs examining relationships between contaminant concentration and site (Table 2), these differences did not translate to detectable differences between reference and spill sites. Greeley et al. (2014) looked at the effects of coal ash contaminated sediments on fathead minnow (*Pimephales promelas*) embryos and larvae and found no adverse effects of coal ash on survival, incidence of developmental abnormalities, or hatching success. Studies of bird nesting around the spill site similarly showed little evidence of physiological impairments, although they had somewhat elevated levels of Se (Beck et al. 2014).

Among the foremost concerns with this ash spill was that long-term, elevated levels of Se may cause reproductive failures or reduced fecundity in aquatic organisms as reported from other Se-contaminated sites (Gillespie and Baumann 1986; Lemly 2002). Fillet Se concentrations were higher at spill sites than reference sites for all three fish species, but this study found little evidence of impending reproductive failure in fishes although there is some evidence of that ash-affected sites experienced some

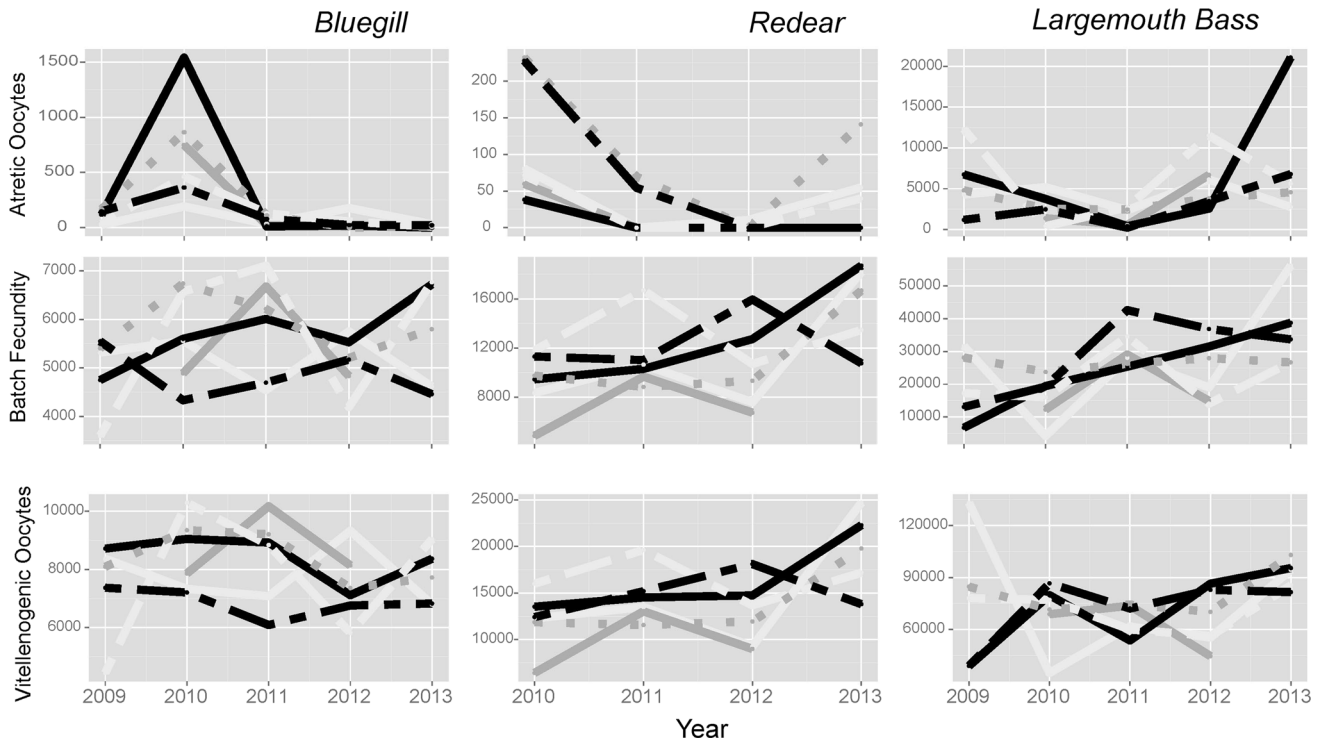

Fig. 4 Time-series of mean number atretic oocytes, mean number vitellogenic oocytes, and mean batch fecundity by site and species. Largemouth bass are not shown because analysis of covariance results showed no significant effect of any metal/metalloid or of site. *Error bars* are omitted for clarity. Legend is as in Fig. 2 and sites are as in Fig. 1

short-term reproductive impairments following the spill. For example, this study reports very low numbers of vitellogenic oocytes in bluegill the spring immediately following the spill at site S1 and higher numbers of atretic oocytes in redear sunfish at sites S2 and S3 in the years following dredging (2010–2011; Fig. 4). Also, there was not a consistent pattern with respect to reference and spill sites with GSI (Table 5; Fig. 3) or significant differences in reproductive metrics at the $\alpha = 0.05$ level for any species (Table 6; Fig. 4), further suggesting that long-term reproductive effects of this coal ash spill were negligible.

It has been documented that coal ash from the TVA KIF spill contains a number of other elements (N = 23, in addition to As, Hg and Se) that can potentially produce toxic effects in fishes such as cobalt, cadmium, and lead. However, due to the high frequency of non-detects and quantities below detection limits, this study focuses solely on As, Hg and Se. Legacy environmental contamination from now-defunct paper mills and other industry in the area has potentially left a variety of other contaminants in the ecosystem that have the potential to influence the fish health response and interact with the class of toxicants that were monitored. Unfortunately, data on non-metal/non-metalloid contaminants, such as polychlorinated biphenyls (PCBs), in fishes were not assessed as part of the TVA KIF monitoring and assessment plan.

The lack of long-term effects of Se on fishes in the current study does not necessarily point to an absence of fish health impacts of the TVA KIF coal ash spill as after effects can take a decade or more to manifest. Selenium accumulates through the food chain, and it may take several years to determine actual trends and effects. For instance, developmental deformities due to Se contamination in Belews Lake, NC did not manifest for 10 years following contamination by coal ash waste inputs as Se was transferred up the food chain from producers to consumers (Lemly 1993). While sediment samples have lower Se than those in Belews Lake, aqueous concentrations were similar between the two systems (Mathews et al. 2014); therefore it's possible that harmful effects of Se have yet to come to light in the Emory and Clinch rivers. However, hydrological differences between the TVA KIF spill area (a lotic ecosystem) and Belews Lake (a lentic ecosystem) may be helping to mitigate effects of the coal ash spill at the TVA KIF plant by continually moving coal ash sediments downstream. It is not possible at this time to know whether the lack of demonstrated fish health impacts from the TVA KIF ash spill is due to insufficient passage of time or to hydrological or other effects thus underscoring the need for continued fish health monitoring in the TVA KIF spill area.

Another hypothesis warranting further examination in this system is that Se concentrations and subsequent health

Table 6 Results for MANCOVAs examining fish reproduction to effects of reproductive metrics (hereafter, metric: atretic oocytes, batch fecundity, vitellogenic oocytes), arsenic (As), mercury (Hg), selenium (Se), fish length (Length), spill (reference or ash-affected sites), year, metrics × spill, metrics × year, year × spill, and metrics × site × year

Species	Effect	Num df	Denom df	F	P
Bluegill	Metric	**2**	**317**	**538.03**	**<0.0001**
	Spill	1	317	0.42	0.5196
	Year	4	317	1.74	0.1401
	Arsenic	1	317	2.31	0.1295
	Mercury	1	317	1.54	0.2149
	Selenium	**1**	317	**11.82**	**0.0007**
	Length	**1**	317	**76.34**	**<0.0001**
	Metric × spill	2	317	2.68	0.0698
	Metric × year	8	317	0.83	0.5754
	Year × spill	**4**	317	**4.58**	**0.0013**
	Metric × spill × year	**8**	317	**3.06**	**0.0025**
Redear	Metric	**2**	**341**	**411.39**	**<0.0001**
	Spill	**1**	**341**	**8.64**	**0.0035**
	Year	3	341	8.34	0.0611
	Arsenic	**1**	**341**	**10.24**	**0.0015**
	Mercury	1	341	1.79	0.1823
	Selenium	**1**	**341**	**10.99**	**0.0010**
	Length	**1**	**341**	**79.66**	**<0.0001**
	Metric × spill	2	341	0.21	0.8093
	Metric × year	**6**	**341**	**5.68**	**<0.0001**
	Year × spill	3	341	2.43	0.0650
	Metric × spill × year	**6**	**341**	**2.40**	**0.0278**
Lg.mouth bass	Metric	**2**	**434**	**208.49**	**<0.0001**
	Spill	1	434	0.09	0.7614
	Year	4	434	2.60	0.0354
	Arsenic	1	434	0.12	0.7316
	Mercury	1	434	0.22	0.6430
	Selenium	1	434	0.11	0.7398
	Length	**1**	434	**52.92**	**<0.0001**
	Metric × spill	2	434	0.09	0.9146
	Metric × year	8	434	1.28	0.2519
	Year × spill	**4**	434	**3.33**	**0.0105**
	Metric × spill × year	8	434	1.05	0.3978

Overall model results are shown in the row with effect "metric" and all results are given as numerator degrees of freedom, denominator degrees of freedom, F-value, and P value. Significant P values ($\alpha = 0.05$) for each covariate from type III sum-of-squares are shown in bold

effects have been much lower in this coal ash spill due to the reported antagonistic interaction between Se and Hg and the abundance of Hg in the study area (Southworth et al. 2000; Sackett et al. 2010). Mercury is a common constituent of coal ash, but compared to legacy sources, there is not conclusive evidence that the ash spill is the major source of Hg (Ruhl et al. 2009). Such interactions may also help to explain why fish tissue concentrations of Se observed near the spill site are substantially lower than those reported following other coal ash spills (e.g., Lohner et al. 2001; Lemly 2002, 2014).

Mercury contamination was generally higher at reference sites than ash-affected sites. In particular, largemouth bass collected from site R1 had levels of Hg that were higher than all other sites (Fig. 2). Potential sources of this Hg at reference sites include a now-closed paper mill upstream of R1 (USEPA 2012) in addition to nearby coal combustion at the TVA KIF site. It is unclear how much of the Hg at ash-affected sites is sourced from the coal-ash spill itself. Bartov et al. (2012) were not able to assign the percentage of Hg from the coal ash spill with certainty from a sediment Hg isotope-speciation study. While some

of the Hg at ash-affected sites appears to be from the coal ash, the exact fraction is not known.

Multigenerational effects of contaminants in other organisms and systems have been shown, so it is possible that adverse effects of the TVA KIF spill have yet to manifest. In particular, long-term studies examining possible effects beyond embryonic and larval stages have not been conducted. There is a growing body of literature showing that effects of chronic metal and metalloid contaminant exposure can vary over time whereby organisms can become increasingly sensitive with successive generations (Stewart et al. 2010; Völker et al. 2013; Jacobasch et al. 2014). For instance, the influence of Hg exposure has been shown to be passed on to subsequent generations (Tsui and Wang 2005; Hammerschmidt and Sandheinrich 2005). The 3–5 years of post-coal ash spill data for a fish species presented in this study may therefore not be enough time to see multigenerational effects. Also, given that the highest contaminant concentrations were recorded in 2010 or 2011 after dredging, when there was only 2 years of data after the highest contaminant concentrations were observed in fish in 2010 (Fig. 2). It is therefore possible that some of the most important fish health and reproductive impacts of the coal ash spill have not yet become apparent or are of sufficiently low level that they are not yet detectible, thus underscoring the importance for continued fish monitoring in the study area.

Conclusions

These types of monitoring studies highlight the desperate need for a more mechanistic understanding surrounding the impact of contaminants on biological systems. Relational studies, as demonstrated here, are a good starting point, but can quickly be overwhelmed by the sheer number of potential combinations and numbers of metrics. Also, as alluded to in this study, effects could be masked by antagonistic interactions that occur at the molecular level which cannot be teased-apart by statistics alone. While there was some suggestion that there may be reproductive effects in redear sunfish, the relatively small sample size and the high degree of individual variability reduced the ability of analyses to detect these differences with higher certainty. Due to this uncertainty and the relational nature of this study, there is a need to follow up this work with carefully designed experiments to determine how adverse effects measured at sub-organism level translate to population effects using an adverse outcome pathway framework (Ankley et al. 2010).

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Compliance with ethical standards

Conflict of interest This study was funded by the Tennessee Valley Authority. All authors declare that they have no conflict of interest

Animal consent All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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