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Species- and stage-specific differences in trace element tissue concentrations in amphibians: implications for the disposal of coal-combustion wastes

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Results suggest that metamorphosed amphibians can transport trace elements from aquatic disposal basins to non-contaminated habitats.

Abstract

Information on species- and stage-specific patterns of contaminant accumulation is generally lacking for amphibians, yet such information could provide valuable knowledge on how amphibians interact with contaminants. We assessed concentrations of As, Cd, Cu, Ni, Pb, Se, Sr, and Zn in whole bodies of larval, recently metamorphosed, and adult life stages in *Bufo terrestris* and *Rana sphenocephala* from a site that currently receives coal combustion waste (CCW) discharge, a site where CCW was formerly discharged that has undergone natural attenuation for 30 years, and a nearby reference site. For the majority of elements (As, Cd, Cu, Ni, Pb, Zn), concentrations were highest in larvae, but Se and Sr concentrations remained elevated in later life stages, likely because these elements are S and Ca analogs, respectively, and are thus retained throughout structural changes during metamorphosis. Element concentrations were generally higher in *B. terrestris* than in *R. sphenocephala*. Concentrations of As, Se, and Sr were up to 11–35 times higher in metamorphs emigrating from CCW-polluted wetlands compared to unpolluted wetlands, suggesting metamorphosed amphibians can transport trace elements from aquatic disposal basins to nearby uncontaminated terrestrial habitats. In addition, anurans utilizing naturally revegetated sites up to 30 years after CCW disposal ceases are exposed to trace elements, although to a lesser degree than sites where CCW is currently discharged.

Keywords: Amphibians; *Bufo terrestris*; Coal combustion wastes; Complex life cycles; Metamorphosis; Natural attenuation; *Rana sphenocephala*; Trace elements

1. Introduction

Environmental pollutants have been hypothesized as a threat to the health of some amphibian populations (Corn, 2000), but identifying species threatened by pollutants is difficult because of differences in their sensitivity to contaminants (Bridges and Semlitsch, 2000; Snodgrass et al., 2004). Variation in species sensitivity to pollutants could be a consequence of numerous factors, including physiological, morphological, ecological, or behavioral differences that influence the extent of exposure to pollutants. Consequently, it is important to identify potential species-specific differences in contaminant accumulation, because such information could help explain variation in species vulnerability to environmental contaminants.

Not only could species vary in their accumulation of environmental contaminants, but the uptake of

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pollutants may also differ among life stages. Most amphibians have complex life cycles, where they undergo metamorphosis from aquatic larvae to semi-aquatic or terrestrial stages. Metamorphosis results in extensive transformations in most aspects of an amphibian's biology, including structural, physiological, behavioral, and ecological changes (Duellman and Trueb, 1994), all of which may influence contaminant accumulation and elimination. For example, larval anurans are thought to experience greater exposure to trace elements compared to other life stages based on differences in feeding habits (Hall and Mulhern, 1984), and their restriction to aquatic habitats where many pollutants are sequestered. Different element retention and elimination patterns in amphibians have also been attributed to the structural reorganization and remodeling of organ systems associated with metamorphosis (Snodgrass et al., 2003). Despite the potential influence of life stage on accumulation of and sensitivity to contaminants in amphibians, to our knowledge, no studies have evaluated intraspecific differences in contaminant accumulation among larva, metamorphic, and adult life stages from the same site.

While an understanding of toxicokinetics in the context of complex life cycles has implications for the health of amphibians, such information may also provide insight into mechanisms by which pollutants may be transported from aquatic to terrestrial systems. Metamorphic amphibians exiting a wetland may number in the tens of thousands (Semlitsch et al., 1996), traveling hundreds of meters into terrestrial habitats (Semlitsch, 1998; Berven and Grudzien, 1990). Because amphibians are important prey and can represent a major portion of the biomass in animal communities (Burton and Likens, 1975), pollutants retained in amphibian tissues through metamorphosis could result in significant transport into the terrestrial food web.

The disposal of coal combustion wastes (CCW) has been identified as a threat potentially contributing to the decline of aquatic-breeding amphibians. Roughly 95 million tons of CCW, which is highly concentrated in trace elements (As, Cd, Cu, Ni, Pb, Se, Sr, Zn, and others), is produced annually in the United States, one-third of which is disposed in aquatic basins where amphibians may breed (Rowe et al., 2002). Numerous species of anurans inhabiting such systems accumulate high concentrations of trace elements and exhibit a variety of adverse responses (Rowe et al., 1996, 1998; Raimondo et al., 1998; Hopkins et al., 1997, 1998, 1999, 2000; Snodgrass et al., 2003, 2004), including complete recruitment failure (Rowe et al., 2001). Not only may aquatic habitats be impacted by such disposal practices but, when not dredged, the basins fill with ash. Once basins fill, a cost-effective approach to site remediation is natural attenuation. When full basins are left to naturally attenuate, terrestrial floral and faunal communities gradually become established. Consequently, amphibians and other wildlife may be exposed to CCW in both aquatic and terrestrial life stages for many years in these contaminated successional habitats (Sample and Suter, 2002).

We conducted a field study to investigate whether trace element accumulation differs between two species of anurans (*Bufo terrestris* and *Rana sphenocephala*) and among their life stages (larva, recent metamorph, adult) at sites contaminated with CCW and a nearby unpolluted site. Although we did not investigate biological effects associated with exposure, our study provides insight into potential species- and stage-specific patterns of contaminant accumulation, as well as information on trace element accumulation at sites where CCW surface discharge and associated contaminants are left to naturally attenuate. Additionally, we assessed whether metamorphic anurans may be vectors of trace element transfer from aquatic to terrestrial food webs, and whether the degree of potential transfer varies by contaminant, species, or site contamination history.

2. Materials and methods

2.1. Study site descriptions

We collected anurans from three sites representing a continuum of CCW pollution history, spanning areas ranging from no known contamination, past contamination and subsequent revegetation, and current discharge of CCW. All study areas are located on the Department of Energy's Savannah River Site in Aiken and Barnwell Counties (SC, USA). The reference site is a preserved, non-impacted area (Department of Energy Set-Aside Area; Davis and Janecek, 1997) that has been a site of long-term studies on old field succession and the ecology of herpetofauna (Odum, 1960; Burke and Gibbons, 1995). At least 24 species of amphibians utilized the reference site during the period of this study (Gibbons, unpubl. data). This site is comprised of a semi-permanent, herbaceous wetland (Carolina Bay No. 176, Ellenton Bay) surrounded by mixed pine/hardwood forest and old field habitats, and has no history of CCW contamination.

A coal-fired power plant on the Savannah River Site (D-Area plant) has been in operation since the early 1950s, but CCW disposal procedures at the site have changed substantially over time. From the 1950s until the late 1960s/early 1970s, the site discharged sluiced CCW into settling basins (retired basins, Fig. 1), which overflowed into a natural depression (i.e., a natural retention basin) in the Savannah River floodplain. The result of this discharge is a plume of CCW (ash plume, Fig. 1) that currently extends up to 2.7 m deep and occupies approximately 40 ha, 30% of which becomes

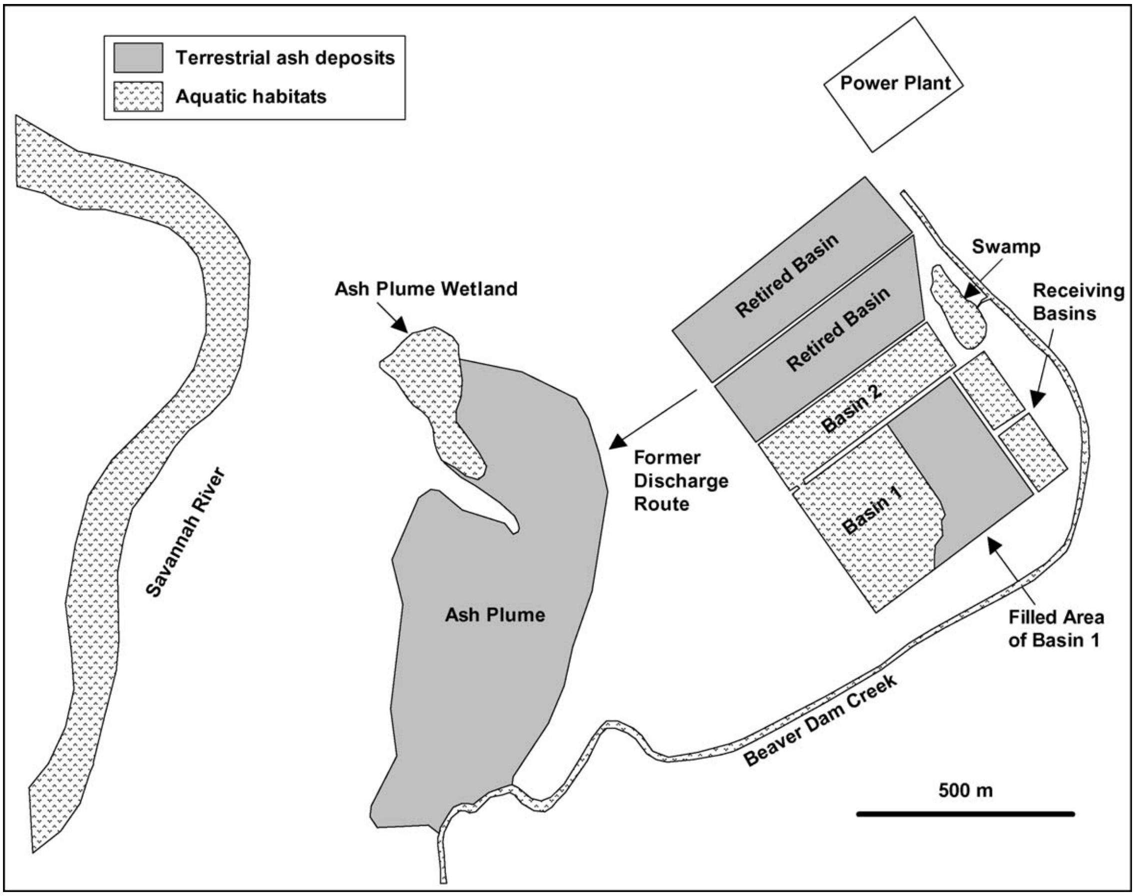

Fig. 1. Map of the coal-burning power plant and disposal sites on the Savannah River Site, SC, USA. The ash plume resulted from coal combustion waste (CCW) overflow from the retired basins and has since undergone revegetation following the cessation of discharge ~30 years ago. Basins 1 and 2 currently receive CCW discharge. Samples were collected from the ash plume wetland and bordering polluted terrestrial areas, and from basin 1. See text for more detailed site descriptions.

occasionally inundated when the Savannah River and Beaver Dam Creek flood. Based on aerial photographs, the majority of vegetation was killed as a consequence of the CCW discharge, but since discharge ceased in the 1970s, a mixed floodplain vegetation community has recolonized. The ash plume now covers a naturally-occurring temporary wetland (perched above the floodplain) and an adjacent bottomland. The forest on the ash plume consists of fairly equal amounts of bald cypress (*Taxodium distichum*), sweetgum (*Liquidambar styraciflua*), and sycamore (*Platanus occidentalis*), followed by red maple (*Acer rubrum*), green ash (*Fraxinus pennsylvvanica*), and loblolly pine (*Pinus taeda*). A thin layer (2.5 cm) of organic material covers the CCW. Based on a recent survey, at least 18 species of amphibians utilize this site (Roe and Hopkins, unpubl. data). Earlier descriptions of the ash plume appear elsewhere (Guthrie and Cherry, 1976; Cherry and Guthrie, 1978).

In the mid-1970s, the power plant shifted CCW discharge into a new series of basins (ash basin; basins 1 and 2, Fig. 1). These basins are open water habitats that currently receive sluiced CCW, with adjacent terrestrial areas recently filled (mid-1980's) with CCW that have begun to revegetate. Vegetation in these terrestrial areas consists primarily of wax myrtle (*Myrica cerifera*), sycamore, cottonwood (*Populus deltoides*), willow (*Salix nigra*), red maple, and sweetgum. At least 19 species of amphibians use this site (Hopkins, unpub. data). Sluiced CCW is pumped from the plant into receiving basins, from which surface water flows into basin 1, basin 2, and then into a swamp before reaching Beaver Dam Creek, a tributary of the Savannah River (Fig. 1). More detailed descriptions of the ash basin system appear elsewhere (Hopkins et al., 1997; Rowe et al., 1998).

2.2. Sample collection and handling

During the spring and summer (March–June) of 2003, we attempted to collect adult, larval, and recently metamorphosed anurans of two species, the southern toad (*Bufo terrestris*) and the southern leopard frog (*Rana sphenocephala*), from the ash plume and reference

Table 1
Sample sizes and number of individuals for each composite sample

Group	Site					
	Reference		Ash plume		Ash basin	
	# of samples	# per sample	# of samples	# per sample	# of samples	# per sample
Bufo terrestris						
Larvae	5	10–13	5	11–13	–	–
Metamorph	5	8	–	–	5	8
Adult[a]	10	1	10	1	–	–
Rana sphenocephala						
Larvae	5	9–10	5	9–11	–	–
Metamorph	12	1	14	1	12	1
Adult[a]	10	1	10	1	–	–

[a] Adult samples include equal number of males and post-oviposition females.

sites (Table 1). We were unsuccessful in capturing recently metamorphosed *B. terrestris* from the ash plume. We collected recent metamorphs of both species from an additional site, the ash basin (basin 1), but this was the only life stage collected from this site (Table 1). Adults (5 male, 5 post-oviposition females of each species; *R. sphenocephala* = 17.4 ± 1.2 g, *B. terrestris* = 16.3 ± 0.9 g; Table 1) that had entered wetlands to breed were collected by hand or with minnow traps. Larvae at stage 25 (Gosner, 1960; *R. sphenocephala* = 0.714 ± 0.039 g, $N = 48$ per site; *B. terrestris* = 0.084 ± 0.003 g, $N = 60$ per site; Table 1) were collected using minnow traps and dip nets. Metamorphosed individuals (*R. sphenocephala* = 3.18 ± 0.13 g, $N = 12–14$ per site; *B. terrestris* = 0.27 ± 0.01 g, $N = 40$ per site; Table 1) were collected on the wetland edge as they exited wetlands. Animals were brought to the laboratory where they were housed in plastic containers containing aged tap water. Animals were held for 48 hours at 25 °C to allow their guts to clear, before being sacrificed and stored at −50 °C until trace element analysis. Animal sizes are presented as mean ± 1 standard error on a wet mass basis.

On 28 April, Plexiglas plates (20 × 25 cm) were suspended in the wetlands at two sites, ash plume and reference, to assess element concentrations in *aufwuchs*, procedurally-defined as the assemblage of organisms, detritus, and sediment that accumulate on submerged surfaces (Newman et al., 1985). At each of these sites, eight plates were suspended vertically just below the surface in water <1 m deep. Two months later, all material was scraped from each plate's surface and dried in an oven (55 °C) prior to analysis. Mean dry mass of *aufwuchs* per plate was 0.36 g (reference) and 0.41 g (ash plume). Element concentrations in *aufwuchs* were not assessed in the ash basin during this investigation, but were determined in a previous study (Rowe et al., 2001). We also collected sediment and water samples from five widely dispersed areas within the wetland at each of the three sites, and eight soil and leaf litter samples from the terrestrial areas within 50 m of the wetland. Sediment, soil, and leaf litter samples were oven-dried (55 °C), and water was filtered (1.2 μm) and acidified before analysis.

2.3. Trace element analysis

Due to the small size of larvae of both species and *B. terrestris* metamorphs, these samples were pooled into groups ranging from 8 to 13 individuals (Table 1). Anuran samples were lyophilized and homogenized prior to trace element analysis. Dried sediments, soil, leaf litter, and *aufwuchs* samples were homogenized before analysis. Concentrations of eight trace elements (As, Cd, Cu, Ni, Pb, Se, Sr and Zn) were determined using an inductively coupled plasma mass spectrometer (Perkin Elmer, Norwalk, CT, USA). We chose this suite of trace elements because they occur in relatively high concentrations in the sediments and biota at CCW-contaminated sites (Rowe et al., 1998; Hopkins et al., 1998, 2000). For a detailed description of digestion and trace element analysis procedures, refer to Hopkins et al. (2004). Mean percent recovery in certified reference materials ranged from 86 to 122%, and mean method detection limits ranged from 0.003 to 0.460 ppm. Concentrations (ppm) are presented as mean ± 1 standard error on a dry mass basis. Water concentrations are in ppb, and minimum detection limits ranged from 0.010 to 0.825 ppb.

2.4. Statistical analyses

The first goal of our analyses was to examine whether element concentrations in environmental media varied among sites. We used a series of MANOVAs to investigate differences in water, sediment, soil, leaf litter, and *aufwuchs* element concentrations among sites, with element concentrations as dependent variables, and site as the independent variable.

The second goal of our analyses was to investigate patterns of trace element accumulation among life stages and between species. For this analysis, we used anurans from the ash plume and reference sites. First, we examined whether element concentrations varied between the sexes only in adults by using a MANOVA with concentrations as dependent variables, and species, sex, site and their interactions as effects. We then combined the sexes and used a MANOVA with species, stage, site, and their interactions as effects.

The final goal of our analyses was to investigate differences in trace element concentrations in a single life stage (metamorphs) exiting wetlands that differ in CCW pollution history. For this analysis, we compared concentrations in metamorphs from all three sites (reference, ash plume, and ash basin) using a MANOVA with species, site, and their interaction as effects.

All element concentrations were $\log_{10}$ transformed prior to analysis to better approximate homoscedasticity and normality. For statistical analyses, we replaced values that were below the instrument's detection limits (BDL) with half of the detection limit of the element. Se concentrations were BDL for seven samples, and Cd concentration was BDL for one sample. We used SAS V 8.1 (SAS Institute, 1999) for all statistical analyses, and designate statistical significance at $P<0.05$.

3. Results

3.1. Study sites

Element concentrations in various components of the wetland habitats differed among sites. Concentrations of all eight elements differed in sediments among sites (MANOVA; site: $\lambda=0.0047$, $F_{16,20}=16.98$, $P<0.001$; Table 2), with most element concentrations highest at ash basin, intermediate in ash plume, and lowest at reference. Pb concentrations were the exception to this trend, with concentrations highest in reference and lowest at ash plume (Table 2). Although the reference site has been protected from human activities for >50 years, it is possible that some undocumented source of Pb existed prior to site protection. *Aufwuchs* element concentrations also differed between reference and ash plume (MANOVA; site: $\lambda=0.0031$, $F_{8,7}=283.09$, $P<0.001$; Table 2). However, only four elements differed significantly between sites, with Zn and Pb highest at reference, and As and Se highest in ash plume (Table 2). Although no statistical comparisons were made, concentrations of most elements in *aufwuchs* at the ash basin site reported by Rowe et al. (2001) were above those from both ash plume and reference (Table 2). Differences in element concentrations in water were only marginally significant among sites (MANOVA; site: $\lambda=0.0006$, $F_{1,8}=193.81$, $P=0.055$; Table 2).

Concentrations of all eight elements in terrestrial habitats adjacent to wetlands differed among sites (MANOVA; soil: $\lambda=0.0019$, $F_{16,28}=38.50$, $P<0.001$; leaf litter: $\lambda=0.0141$, $F_{16,28}=13.01$, $P<0.001$; Table 2). Soil concentrations were higher in both CCW-polluted habitats relative to reference, and generally highest at ash basin, but the two CCW-polluted habitats differed little in Ni, Zn, and Sr concentrations (Table 2). Concentrations of most elements in leaf litter were highest at ash basin, intermediate at ash plume, and

Table 2
Concentrations of trace elements from wetland (sediment, *aufwuchs*, and water) and surrounding upland (soil, leaf litter) habitats at three sites in South Carolina, USA

	As	Cd	Cu	Ni	Se	Sr	Pb	Zn
Sediment								
Reference	1.3±0.5	0.2±0.1	12.3±2.8	6.0±0.9	1.3±0.2	11.1±2.2	43.3±11.4	24.7±5.6
Ash Plume	70.2±13.5	0.3±0.1	43.1±5.4	24.0±2.2	10.1±1.4	181.9±26.2	17.2±3.7	30.8±6.7
Ash Basin	140.0±16.6	1.5±0.3	83.4±5.7	34.5±3.7	10.7±1.1	192.7±36.8	24.7±1.9	47.8±3.6
Aufwuchs								
Reference	12.2±1.4	0.9±0.1	18.5±2.2	14.7±4.5	0.4±0.1	25.7±5.2	23.8±4.9	113.9±20.1
Ash Plume	122.9±37.9	1.0±0.1	15.7±1.3	7.4±0.3	8.4±0.2	20.1±1.5	10.6±0.5	21.4±1.8
Ash Basin[a]	140.8±4.9	20.1±1.2	550.1±25.6	356.7±23.9	11.9±0.1	163.7±10.5	21.6±0.6	1,161.0±66.0
Water								
Reference	4.1±0.4	0.06±0.02	1.8±0.3	1.3±0.3	0.4±0.1	21.2±4.2	1.37±0.14	161.5±28.0
Ash Plume	2.0±0.1	BDL	0.9±0.1	11.7±0.5	BDL	172.8±6.9	0.11±0.04	158.8±8.9
Ash Basin	21.9±12.8	1.16±0.26	23.8±3.0	8.3±0.7	1.3±0.4	156.1±51.9	0.02±0.01	53.4±5.4
Soil								
Reference	1.3±0.1	0.2±0.01	4.6±0.4	2.1±0.2	0.09±0.04	3.2±0.1	7.9±1.9	11.2±0.8
Ash Plume	33.5±4.1	0.3±0.04	33.9±2.2	27.0±1.5	3.8±0.4	206.4±21.1	15.9±1.3	39.5±4.4
Ash Basin	86.3±5.0	1.3±0.4	53.2±3.2	28.6±3.4	7.4±0.5	237.2±57.0	24.7±1.5	36.8±1.9
Leaf litter								
Reference	0.8±0.1	0.3±0.1	9.5±0.8	3.1±0.5	0.2±0.1	46.1±7.3	3.4±0.2	46.9±6.3
Ash Plume	6.7±3.6	0.5±0.1	14.8±1.4	10.1±1.3	3.3±0.7	443.7±63.8	2.8±0.6	52.5±9.1
Ash Basin	12.1±2.9	1.5±0.4	22.4±2.5	14.0±1.0	19.0±2.1	502.6±67.4	5.7±1.1	114.5±23.1

The reference site has no history of CCW pollution, the ash plume formerly received CCW, and CCW is currently discharged into the ash basin site. Water concentrations are ppb, and all others are ppm dry mass. Note: BDL = below detection limit.

[a] *Aufwuchs* concentrations in the ash basin are from Rowe et al. (2001).

lowest at reference. Concentrations of Zn, Cd, and Pb were similar at reference and ash plume, and Sr concentrations were similar at ash plume and ash basin (Table 2).

3.2. Life stage and species comparisons

Overall, sex influenced element concentrations in adults, but differences between the sexes depended upon species (MANOVA; sex: $\lambda = 0.539$, $F_{8,25} = 2.67$, $P = 0.029$, species×sex: $\lambda = 0.392$, $F_{8,25} = 4.85$, $P = 0.001$). The effect of sex was evident only for Cu and Pb. Males of both species had higher Cu concentrations than females at both reference and ash plume (reference: *R. sphelocephala*, 9.5 ± 2.2 (M), 6.1 ± 1.1 (F), *B. terrestris*, 15.7 ± 2.9 (M), 12.9 ± 3.0 (F); ash plume: *R. sphenocephala*, 10.0 ± 2.3 (M), 7.7 ± 1.3 (F), *B. terrestris*, 31.6 ± 12.8 (M), 12.0 ± 1.2 (F)). *R. sphenocephala* females had higher Pb concentrations than males (reference: 0.6 ± 0.2 (M), 1.3 ± 0.3 (F); ash plume: 0.2 ± 0.1 (M), 0.4 ± 0.1 (F)) but Pb concentrations were similar between the sexes in *B. terrestris*. Because we failed to detect large and consistent differences between the sexes, we grouped males and females for other analyses that focused on differences attributable to site, species, and lifestage.

From our analysis of anurans at the ash plume and reference, element concentrations differed among life stages, but differences depended upon site, species, and element (Table 3, Fig. 2). In the ash plume, As, Cd, Cu, Ni, Pb, and Zn were generally higher in larvae than in metamorphs and adults. Differences among life stages for these elements were less evident at reference, with the exception of Pb, which was highest in larvae relative to other stages (Fig. 2). Patterns of accumulation of Se and Sr among life stages differed from those of other elements (Fig. 2). In the ash plume, larval and metamorphic *R. sphenocephala* had elevated Se concentrations compared to adults, which had Se concentrations comparable to conspecifics at reference. There were no differences in Se concentrations among *R. sphenocephala* life stages at reference. For *B. terrestris*, life stage had little influence on Se concentrations at either site, but Se concentrations were higher at ash plume compared to reference in all life stages. Sr concentrations increased with developmental stage (larva < metamorph < adult) in both species at each site, but differences among life stages at ash plume were more extreme compared to reference (Fig. 2).

Table 3
Results of multivariate analysis of variance of the effects of species, life stage, and site on the concentration of eight trace elements in the anurans *Bufo terrestris* and *Rana sphenocephala*

Source	Test statistic (λ)	df	F	P
Species	0.7041	8, 73	3.83	<0.001
Stage	0.0174	16, 146	60.11	<0.001
Species × stage	0.4101	16, 146	5.12	<0.001
Site	0.3001	8, 73	21.20	<0.001
Species × site	0.8296	8, 73	1.87	0.077
Stage × site	0.2485	16, 146	9.18	<0.001
Species × stage × site	0.7320	8, 73	3.34	0.003

For As, Cd, Cu, Ni, Pb, and Zn, *B. terrestris* larvae accumulated concentrations 2× higher than *R. sphenocephala* at the ash plume. Differences between species were less evident for larvae at reference and for metamorphs and adults at either site, although Ni concentrations were higher in larval *B. terrestris* than *R. sphenocephala* at the reference site, while Pb concentrations were higher in larval *R. sphenocephala* than *B. terrestris* at reference. For Se and Sr, patterns of accumulation between species were different from those of other elements (Fig. 2). Se and Sr concentrations in larvae of both species were similar within each site, but differences between species were evident in adults at ash plume, where *B. terrestris* accumulated concentrations of both Se and Sr over 2× higher than *R. sphenocephala*. Both species had similar concentrations of Se and Sr at reference in all life stages.

3.3. Comparison of metamorphs

From our analysis of metamorphs emigrating from wetlands at the reference, ash plume, and ash basin sites, tissue element concentrations differed between species, but the degree of differences between species depended upon site (MANOVA; species: $\lambda = 0.1325$, $F_{8,36} = 29.44$, $P < 0.001$; site: $\lambda = 0.0025$, $F_{16,72} = 84.16$, $P < 0.001$; site×species: $\lambda = 0.1238$, $F_{8,36} = 31.86$, $P < 0.001$). There was no uniform trend in patterns of contaminant accumulation between species and among sites in metamorphs; rather, patterns of contaminant accumulation depended upon the particular element (Fig. 3).

For As, Se, Sr, Cu, and Cd, there was a general trend for metamorphs exiting the ash basin to have the highest concentrations, those at reference to have the lowest, and with the exception of Cd, metamorphs from ash plume were intermediate. Concentrations of Se in metamorphs of both species were similar within each site. Concentrations of As, Sr, Cd, and Cu in metamorphs were similar between species at reference, but while both species had elevated concentrations of As and Sr at ash basin, concentrations of As and Sr in *B. terrestris* were higher relative to *R. sphenocephala*. The opposite was true for Cu and Cd, with concentrations higher in *R. sphenocephala* than *B. terrestris* at ash basin (Fig. 3).

Concentrations of Ni, Zn, and Pb in metamorphs exhibited different patterns from the other elements and one another. For Ni, concentrations were lowest in *R. sphenocephala* metamorphs from ash plume, but similar at the other two sites in both species. For Zn, there was little difference between species at reference. However, among the three sites, Zn concentrations in *R. sphenocephala* were lowest at ash basin but highest in

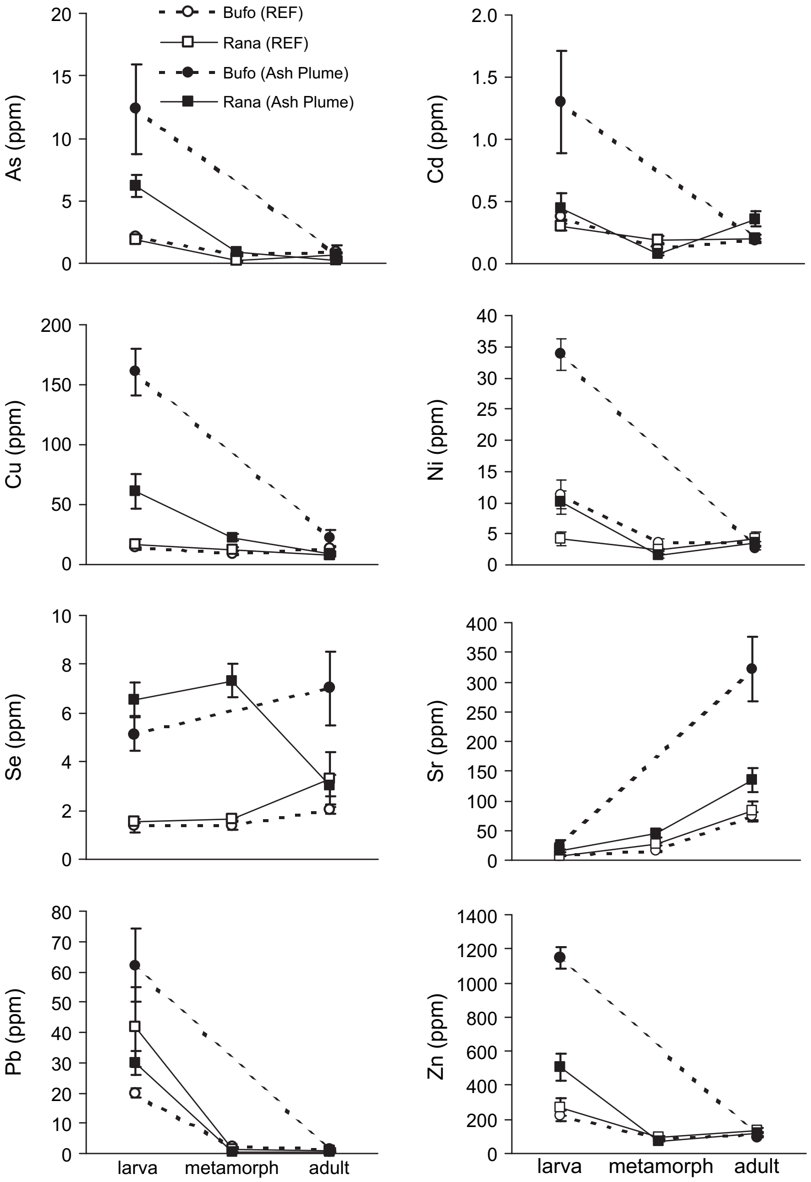

Fig. 2. Trace element concentrations (ppm dry mass) in *Bufo terrestris* (*Bufo*) and *Rana sphenocephala* (*Rana*) larvae, metamorphs, and adults from a site where coal combustion wastes (CCW) were formerly discharged (ash plume) and a reference site (REF) with no history of CCW pollution. Lines connecting the points do not imply samples were related, but are drawn to elucidate patterns of element concentrations of each group. We were unsuccessful in collecting *B. terrestris* metamorphs from the ash plume. Values are means ± 1 standard error. Note the differences in *y*-axis scales.

B. terrestris at this site. Finally, Pb concentrations were highest at reference and lowest at ash basin, and higher in *B. terrestris* compared to *R. sphenocephala* (Fig. 3).

4. Discussion

4.1. Stage-specific differences

The transformations associated with complex life cycles in anurans can greatly influence trace element uptake and elimination patterns, yet few studies have directly evaluated the influence of life stage on contaminant accumulation. Most studies focus on a single life stage, usually larvae, but our study is unique in that we investigate intraspecific patterns of element concentrations across life stages representative of a species' complete life cycle, with the exception of the embryo. Concentrations of most elements were highest in larvae, supporting the contention that the larval stage in amphibians may receive the greatest exposure to trace elements (Hall and Mulhern, 1984; Brisdall et al., 1986; Grillitsch and Chovanec, 1995). Variation in element accumulation among life stages likely relates to a combination of factors, including different feeding habits, habitat use, and movements between aquatic larvae and

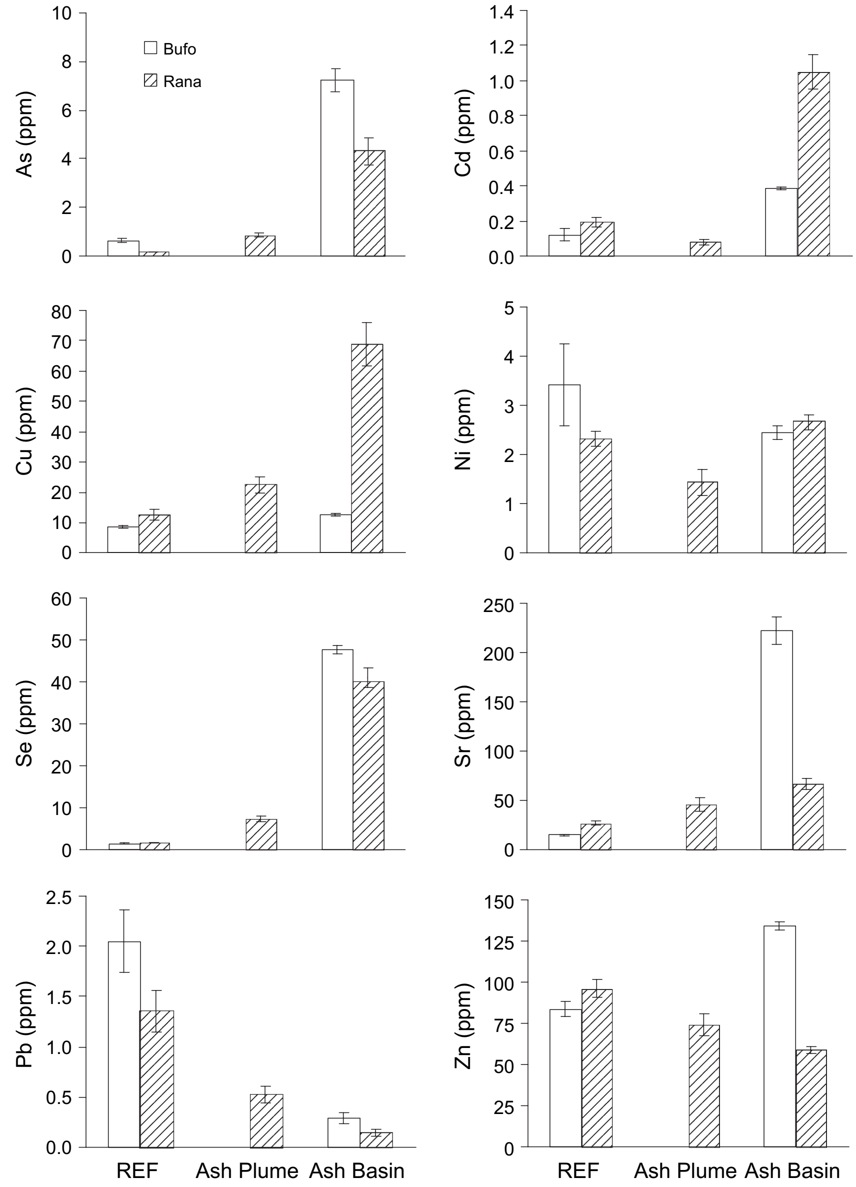

Fig. 3. Trace element concentrations (ppm dry mass) in *Bufo terrestris* (*Bufo*) and *Rana sphenocephala* (*Rana*) metamorphs from a site historically polluted with coal combustion wastes (CCW) that has undergone revegetation (ash plume), a site where CCW is currently discharged (ash basin), and a reference site (REF) with no history of CCW pollution. We were unsuccessful in collecting *B. terrestris* metamorphs from the ash plume. Values are means ± 1 standard error. Note the differences in *y*-axis scales.

terrestrial juveniles and adults. Most anuran larvae are microphagous, omnivorous filter or scraper feeders and while feeding they ingest large quantities of sediment, where most elements are concentrated in polluted aquatic systems (Guthrie and Cherry, 1976). Because most are omnivores, anuran larvae typically have a large intestinal surface area for their body size (Duellman and Trueb, 1994; Hourdry et al., 1996), which likely enhances contaminant absorption. In contrast, juveniles and adults are carnivorous, rarely ingesting large quantities of soil or sediment during feeding, and the length of the intestine is relatively short compared to larvae (Hourdry et al., 1996). Element bioavailability and mobility may also differ between terrestrial and aquatic environments. Anuran larvae are restricted to aquatic habitats where many pollutants are sequestered, whereas terrestrial juveniles and adults may move between areas of high and low contamination (Hopkins et al., 1998). Although concentrations of many trace elements in soil and leaf litter immediately adjacent to the wetland did not differ greatly from that in the wetland sediment (Table 2), uncontaminated terrestrial areas at our polluted study site are 0–75 meters from the wetland, which is within the typical dispersal distances of amphibians (Berven and Grudzien, 1990; Semlitsch, 1998; Fig. 1).

Differences in element concentrations between aquatic larvae and terrestrial life stages may also be a consequence of element elimination and retention during metamorphosis. During metamorphosis, anurans do not feed as they undergo extensive physiological and morphological changes (Hourdry et al., 1996). Consequently, element concentrations in recently metamorphosed anurans are largely dependent upon previous larval accumulation and patterns of elimination and retention throughout metamorphosis. Of particular importance to understanding stage-specific accumulation is the restructuring of the digestive system and other organ systems during metamorphosis. Concentrations of As, Cd, Cu, Ni, Pb, and Zn decreased from the larval to metamorphic stage of both species at ash plume (Fig. 2), and with the exception of Zn, similar decreases were also observed at metamorphosis in *R. catesbeiana* (Snodgrass et al., 2003). These elements concentrate in the digestive tract and other organs of larvae (Sparling and Lowe, 1996; Burger and Snodgrass, 1998), and are thus likely to be eliminated or redistributed in the body during remodeling of the gut and other organ systems. Additionally, because anurans cease feeding during metamorphosis, elimination may exceed uptake rates. The above combination of factors could contribute to lower concentrations of these elements in metamorphs.

While concentrations of most elements were lowest in metamorphs and adults, concentrations of Se and Sr in terrestrial stages were greater than or equal to concentrations in larvae in some cases (Fig. 2). Likewise, Se and Sr were retained through metamorphosis in *R. catesbeiana* and *R. clamitans* (Snodgrass et al., 2003, 2004), suggesting the properties of these elements and their partitioning within the body differ from other elements. Indeed, Se concentrations in the carcass (minus the digestive tract) were higher than gut concentrations in larval *R. catesbeiana* (Burger and Snodgrass, 1998), suggesting its retention is due to incorporation into tissues other than the digestive tract. Se and Sr are S and Ca analogs, respectively, and may thus be incorporated into tissues, such as protein and bone (Masscheleyn and Patrick, 1993; Snodgrass et al., 2003), allowing these elements to be retained throughout metamorphosis. Although Se is retained in recently metamorphosed individuals, the fate of Se in adults is less clear. The lower Se concentrations in adult *R. sphenocephala* suggest Se may be eventually eliminated or diluted as the animal grows, but Se concentrations observed in *B. terrestris* adults indicate continued exposure to and accumulation of Se in the terrestrial habitat. Differences in site fidelity and/or feeding ecology may also contribute to differences in adults of the two species. Such differences suggest that not only stage, but also species-specific traits play important roles in amphibian interactions with pollutants.

4.2. Species-specific differences

With the exception of Se, for all life stages where individuals from polluted sites had elevated element concentrations relative to reference sites, accumulation was >2× higher in *B. terrestris* than in *R. sphenocephala* (Fig. 2). Few studies have investigated element accumulation among anuran species simultaneously at the same polluted site, but Sparling and Lowe (1996) found significant variation in element concentrations between larval *Acris crepitans* and *Hyla versicolor*. Additional evidence of species-specific patterns of element accumulation comes from comparisons between *R. clamitans* and *R. sylvatica* (Snodgrass et al., 2004), *R. catesbeiana* and *R. sphenocephala* (Burger and Snodgrass, 2001), and among *B. bufo*, *R. dalmatina*, and *R. ridibunda* (Grillitsch and Chovanec, 1995), although variation reported in these investigations was complicated by different exposure conditions, sampling times, or sites.

The biological significance of differences in element accumulation has not been explored. Evidence from other studies suggests the effects of CCW pollution may be more substantial in *B. terrestris* larvae compared to *R. catesbeiana*, a congener of *R. sphenocephala*. Numerous lethal and sublethal effects have been documented in *B. terrestris*, including hormonal responses (Hopkins et al., 1997, 1999) and complete larval mortality (Rowe et al., 2001). In the current study, no metamorphic *B. terrestris* were found at one of the CCW-polluted sites, and despite a large breeding aggregation at this site, *B. terrestris* tadpoles were in lower density than *R. sphenocephala* based on relative capture efforts. Compared to the complete larval mortality in *B. terrestris* observed by Rowe et al. (2001), a larger proportion of *R. catesbeiana* survived (15%) in a similar study, but a suite of sublethal effects has been documented in several Ranid species exposed to CCW (Rowe et al., 1996, 1998; Raimondo et al., 1998; Hopkins et al., 2000; Snodgrass et al., 2004). Whether *B. terrestris* is more susceptible than Ranids to CCW as a direct result of its higher accumulation of contaminants requires further study.

We can only speculate on the mechanistic basis for variation in element accumulation in *B. terrestris* and *R. sphenocephala*. It is possible that such differences stem from variation in microhabitat use (Sparling and Lowe, 1996), feeding behavior, diet, and associated gut characteristics. Larvae of different species often vary in gut morphology and intestinal material, with primarily herbivorous species having large gut area to body length ratios, and bottom feeding species passing more inorganic material through the gut (Altig and Kelly, 1974). Indeed, higher element concentrations in *B. bufo* were associated with higher gut to carcass ratios compared to *R. dalmatina* (Grillitsch and Chovanec, 1995), suggesting species with a proportionally larger gut would likely have higher whole body contaminant concentrations, but such measurements for *B. terrestris* and *R. sphenocephala* were beyond the scope of our investigation. Alternatively, variation in element accumulation may be a consequence of larval body size differences, since *B. terrestris* is considerably smaller than *R. sphenocephala*. Uptake and elimination patterns often depend on a complex combination of factors that scale with body size including surface area to volume ratios, metabolism, ingestion rate, growth efficiency, and elimination rate (Newman and Heagler, 1991). Exposure duration could also influence accumulation, but the observed accumulation patterns are inconsistent with this possibility; *B. terrestris* has a shorter larval period than *R. sphenocephala* in our study area (Martof et al., 1980).

4.3. Implications for the management of coal combustion wastes

Although whole body concentrations of most elements decreased during metamorphosis, anurans metamorphosing from polluted wetlands still retained high levels of several elements. In particular, concentrations of As and Se in metamorphs of both species, as well as Sr in *B. terrestris*, were an order of magnitude higher at the ash basin compared to conspecifics at reference (Fig. 3). Thus, even elements such as As, which decrease in concentration during metamorphosis, remain elevated in metamorphs exiting polluted wetlands. Adult and larval amphibians are not especially mobile, and it is this trait that presumably limits their role as agents of spatial nutrient transfer (Burton and Likens, 1975). However, immediately following metamorphosis, pond-breeding amphibians can disperse hundreds of meters into terrestrial habitats (Berven and Grudzien, 1990; Semlitsch, 1998). Through such movements, contaminants accumulated during the larval stage may be transferred by metamorphic amphibians to otherwise uncontaminated terrestrial food webs (Snodgrass et al., 2003).

Allowing CCW disposal sites to naturally revegetate is the least costly remediation action once they fill with ash, yet little is known about the fate of contaminants and impacts on biota in these revegetated systems. To our knowledge, this is the first investigation of contaminant accumulation in amphibians inhabiting retired CCW surface disposal basins. In the only other study where contaminant levels were examined in retired CCW disposal basins, small mammals and deer accumulated potentially toxic concentrations of As and Se (Sample and Suter, 2002). In our study, anurans inhabiting sites historically polluted with CCW that have undergone subsequent revegetation are still exposed to trace elements long after CCW disposal ceases (up to 30 years), although to a lesser degree than sites where CCW is currently discharged. For example, concentrations of four elements (As, Cu, Se, and Sr) in metamorphic *R. sphenocephala* from ash plume were significantly (1.7–4.8×) higher than those at reference but below those at ash basin (Fig. 3). A number of natural physical, chemical, and biological processes, including export by emigrating biota (Cherry and Guthrie, 1978) and changes in contaminant bioavailability and mobility may lead to natural attenuation of trace elements over time in these retired systems, but how long these contaminants remain available to biota remains in question.

Although we did not measure biological effects of CCW, we identified important stage- and species-specific patterns of trace element accumulation that contribute to our understanding of how amphibians interact with contaminants. In particular, species- and stage-specific sensitivities may be in part related to species- and stage-specific patterns of accumulation, although numerous other factors influence sensitivity. Our findings also have implications for the management of CCW. Particular elements accumulated by larvae are retained in metamorphs, a life stage that can mobilize trace elements from aquatic disposal basins to nearby uncontaminated terrestrial habitats and serve as vectors of food chain contamination. For at least one element, Se, concentrations in metamorphs of both species (40–50 ppm) were comparable to dietary Se concentrations known to adversely affect growth, reproduction, and survival in predatory birds (reviewed in Ohlendorf, 2003). Consequently, the impacts of aquatic CCW disposal may extend beyond the boundaries of these industrial sites. Although further study of additional sites is necessary, our data suggest remediation strategies that rely on natural attenuation of trace elements do not eliminate uptake of contaminants by amphibians at these sites up to 30 years after CCW disposal ceases.

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References

Altig, R., Kelly, J.P., 1974. Indices of feeding in anuran tadpoles as indicated by gut characteristics. Herpetologica 30, 200–203.

Berven, K.B., Grudzien, T.A., 1990. Dispersal in the wood frog (*Rana sylvatica*): implications for genetic population structure. Evolution 44, 2047–2056.

Bridges, C.M., Semlitsch, R.D., 2000. Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. Conservation Biology 14, 1490–1499.

Brisdall, C.W., Grue, C.E., Anderson, A., 1986. Lead concentrations in bullfrog *Rana catesbeiana* and green frog *R. clamitans* tadpoles inhabiting highway drainages. Environmental Pollution A 40, 233–247.

Burger, J., Snodgrass, J., 1998. Heavy metals in bullfrog (*Rana catesbeiana*) tadpoles: effects of depuration before analysis. Environmental Toxicology and Chemistry 17, 2203–2209.

Burger, J., Snodgrass, J., 2001. Metal levels in southern leopard frogs from the Savannah River Site: location and body compartment effects. Environmental Research A 86, 157–166.

Burke, V.J., Gibbons, J.W., 1995. Terrestrial buffer zones and wetland conservation: a case study of freshwater turtles in a Carolina Bay. Conservation Biology 9, 1365–1369.

Burton, T.M., Likens, G.E., 1975. Salamander populations and biomass in the Hubbard Brook Experimental Forest, New Hampshire. Copeia 1975, 541–546.

Cherry, D.S., Guthrie, R.K., 1978. Mode of elemental dissipation from ash basin effluent. Water, Air, and Soil Pollution 9, 403–412.

Corn, P.S., 2000. Amphibian declines: review of some current hypotheses. In: Sparling, D.W., Linder, G., Bishop, C.A. (Eds.), Ecotoxicology of Amphibians and Reptiles. Society of Environmental Toxicology and Chemistry Press, Pensacola, FL, pp. 663–696.

Davis, C.E., Janecek, L.L., 1997. Departement of Energy Research Set-Aside Areas of the Savannah River Site. Savannah River Site National Environmental Research Park Program, United States Departement of Energy, Publication # SRO-NERP-25.

Duellman, W.E., Trueb, L., 1994. Biology of amphibians. John's Hopkins University Press, Baltimore, MD.

Gosner, K.L., 1960. A simplified table for staging anuran embryos and larva with notes on identification. Herpetologica 16, 183–190.

Grillitsch, B., Chovanec, A., 1995. Heavy metals and pesticides in anuran spawn and tadpoles, water, and sediment. Toxicological and Environmental Chemistry 50, 131–155.

Guthrie, R.K., Cherry, D.S., 1976. Pollutant removal from coal-ash basin effluent. Water Resources Bulletin 12, 889–902.

Hall, R.J., Mulhern, B.M., 1984. Are anurans heavy metal accumulators? In: Seigel, R.A., Hunt, L.E., Knight, L.M., Zuschlag, N.L. (Eds.), Vertebrate Ecology and Systematics – a Tribute to Henry S. Fitch Museum of Natural History, University of Kansas, Lawrence, KS, pp. 123–133.

Hopkins, W.A., Mendonca, M.T., Congdon, J.D., 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion waste. General and Comparative Endocrinology 108, 237–246.

Hopkins, W.A., Mendonca, M.T., Rowe, C.L., Congdon, J.D., 1998. Elevated trace element concentrations in southern toads, *Bufo terrestris*, exposed to coal combustion wastes. Archives of Environmental Contamination and Toxicology 35, 325–329.

Hopkins, W.A., Mendonca, M.T., Congdon, J.D., 1999. Responsiveness of the hypothalamo-pituitary-interrenal axis in an amphibian (*Bufo terrestris*) exposed to coal combustion wastes. Comparative Biochemistry and Physiology C 122, 191–196.

Hopkins, W.A., Congdon, J.D., Ray, J.K., 2000. Incidence and impact of axial malformations in bullfrog larvae developing in sites impacted by coal combustion byproducts. Environmental Toxicology and Chemistry 19, 862–868.

Hopkins, W.A., Staub, B.P., Baionno, J.A., Jackson, B.P., Roe, J.H., Ford, N.B., 2004. Trophic and maternal transfer of selenium in brown house snakes (*Lamprophis fuliginosus*). Ecotoxicology and Environmental Safety 58, 285–293.

Hourdry, J., L'Hermite, A., Ferrand, R., 1996. Changes in the digestive tract and feeding behavior of anuran amphibians during metamorphosis. Physiological Zoology 69, 219–251.

Martof, B.S., Palmer, W.M., Bailey, J.R., Harrison, J.R., 1980. Amphibians and Reptiles of the Carolina's and Virginia. The University of North Carolina Press, Chapel Hill, NC.

Masscheleyn, P.H., Patrick, W.H., 1993. Biogeochemical processes affecting selenium cycling in wetlands. Environmental Toxicology and Chemistry 12, 2235–2243.

Newman, M.C., Heagler, M.G., 1991. Allometry of metal bioaccumulation and toxicity. In: Newman, M.C., McIntosh, A.W. (Eds.), Metal Ecotoxicology. Concepts and Applications. Lewis Publishers, Chelsea, MI, pp. 99–130.

Newman, M.C., Alberts, J.J., Greenhunt, V.A., 1985. Geochemical factors complicating the use of *aufwuchs* to monitor bioaccumulation of arsenic, cadmium, chromium, copper, and zinc. Water Research 19, 1157–1165.

Odum, E.P., 1960. Organic turnover and production in old field succession. Ecology 41, 34–49.

Ohlendorf, H.M., 2003. Ecotoxicology of selenium. In: Hoffman, D.J., Rattner, B.A., Burton, G.A., Cairns, J. (Eds.), Handbook of Ecotoxicology. Lewis Publishers, Boca Raton, FL, pp. 465–500.

Raimondo, S.M., Rowe, C.L., Congdon, J.D., 1998. Exposure to coal ash impacts swimming performance and predator avoidance in larval bullfrogs (*Rana catesbeiana*). Journal of Herpetology 32, 289–292.

Rowe, C.L., Kinney, O.M., Fiori, A.P., Congdon, J.D., 1996. Oral deformities in tadpoles (*Rana catesbeiana*) associated with coal ash deposition: effects on grazing ability and growth. Freshwater Biology 36, 723–730.

Rowe, C.L., Kinney, O.M., Nagle, R.D., Congdon, J.D., 1998. Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. Physiological Zoology 71, 27–35.

Rowe, C.L., Hopkins, W.A., Coffman, V.R., 2001. Failed recruitment of southern toads (*Bufo terrestris*) in a trace element-contaminated breeding habitat: direct and indirect effects that may lead to a local population sink. Archives of Environmental Contamination and Toxicology 40, 399–405.

Rowe, C.L., Hopkins, W.A., Congdon, J.D., 2002. Ecotoxicological implications of aquatic disposal of coal combustion residues in the United States: a review. Environmental Monitoring and Assessment 80, 207–276.

Sample, B.E., Suter, G.W., 2002. Screening evaluation of the ecological risks to terrestrial wildlife associated with a coal ash disposal site. Human and Ecological Risk Assessment 8, 637–656.

SAS Institute, 1999. SAS Users guide: Statistics. Version 8. SAS Institute, Cary, NC.

Semlitsch, R.D., 1998. Biological delineation of terrestrial buffer zones for pond-breeding salamanders. Conservation Biology 12, 1113–1119.

Semlitsch, R.D., Scott, D.E., Pechmann, J.H.K., Gibbons, J.W., 1996. Structure and dynamics of an amphibian community: evidence from a 16-year study of a natural pond. In: Cody, M., Smallwood, J. (Eds.), Long Term Studies of Vertebrate Communities. Academic Press, San Diego, CA, pp. 217–248.

Snodgrass, J.W., Hopkins, W.A., Roe, J.H., 2003. Relationships among developmental stage, metamorphic timing, and concentrations of elements in bullfrogs (*Rana catesbeiana*). Environmental Toxicology and Chemistry 22, 1597–1604.

Snodgrass, J.W., Hopkins, W.A., Broughton, J., Gwinn, D., Baionno, J.A., Burger, J., 2004. Species-specific responses of developing anurans to coal combustion wastes. Aquatic Toxicology 66, 171–182.

Sparling, D.W., Lowe, T.P., 1996. Metal concentrations of tadpoles in experimental ponds. Environmental Pollution 19, 149–150.