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Toxicity of metal-enriched black shale-draining surface waters to *Ceriodaphnia dubia*, and *Pimephales promelas*

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Abstract

Metal-contaminated surface waters can result in adverse effects upon aquatic organisms leading to declines in taxa richness and abundance, and shifts of community composition due to elimination of metal-sensitive taxa. We measured the concentrations and spatial distribution of naturally-derived metals in surface waters collected from four creeks in north-central Arkansas, USA. Three of these creeks flow over the Mississippian Fayetteville Shale (Trace, Begley, and Cove creeks), whereas the fourth one (Mill Creek) flows over the Mississppian Pitkin Limestone. We also evaluated the potential impacts of metals upon aquatic organisms by conducting standardized toxicity tests using *Ceriodaphnia dubia* and *Pimephales promelas*. Water hardness (expressed as $CaCO_3$), dissolved organic carbon (DOC), and Ni, Cd, Cu, and Pb concentrations were significantly higher in Trace, Begley, and Cove creeks than Mill creek ($p < 0.001$). Neonate production in the control and Mill creek treatments were significantly higher than that of Trace, Begley, and Cove creeks ($p < 0.05$). Survival and growth of *P. promelas* larvae (< 24-h) in Trace, Begley, and Cove creeks were significantly lower than that of Mill creek or the control ($p<0.05$). The degree of relative impact of metal-enriched black shale-draining stream waters upon aquatic communities was dependent upon the bioavailable metal fraction, which in part was attributed to competition for binding sites between Ca^{2+} and the cationic metals, and metal complexation with DOC ligands. Our results suggested that aquatic organisms in shale-draining creeks were exposed to elevated metal concentrations that caused the observed lethal and sublethal effects upon the fathead minnow and waterflea. However, further studies on metal bioaccumulation and community response to metal enrichment are needed to disclose the specific impacts of shale-derived metals on the resident biota.

Key words: Black shales, trace metals, toxicity, metal bioavailability, Little Red River, Arkansas, *Ceriodaphina dubia, Pimephales promelas*.

Introduction

Metals in surface waters consist of inputs from natural geochemical processes and anthropogenic activities. Natural geochemical processes are significant non-point sources of metal pollution in surface waters that pose enormous challenges to management and regulatory agencies. Despite the focus on the sources and impacts of metals originating from anthropogenic activities (Norberg-King et al., 1991; Sarakinos et al., 1999; Bailey et al., 2000; Van Sprang & Janssen, 2001), recent studies have demonstrated that natural geochemical materials, such as black shales, may be enriched with potentially toxic metals (Coveney & Glassock, 1989; Kim & Thornton 1993; Chon et al., 1996; Lee et al., 1998; Petsch, 2002; Ogendi et al., 2004b). However, there is scant information on the impacts of natural or black shale-sourced metals on aquatic biota. Thus, a study on the potential effects of these metals will better estimate environmental risk associated with their elevated concentrations in surface waters. This study evaluated the ability of surface waters draining over black shales and containing elevated metal concentrations, to cause mortality, growth, and reproductive impairments on aquatic biota.

Metals in water and / or sediments have the potential of causing deleterious effects upon aquatic organisms (Parametrix, 1995; Van Sprang & Janssen, 2001). They may cause lethal and sublethal effects upon the resident biota which in turn may lead to declines in taxa richness and abundance, and shifts of community composition due to elimination of metal-sensitive taxa within the affected aquatic ecosystems (Clements, 1994; David, 2003; Van Griethuysen et al., 2004). Hickey and Clements (1998) observed a reduced abundance of metal-sensitive mayflies and an increase in metal-tolerant orthoclad chironomids in New Zealand streams. Macroinvertebrates tolerate elevated metal concentrations by accumulating them in metal binding proteins thereby limiting their interaction with the cells. Others tolerate metal contamination through efficient detoxification mechanisms, and storage of metals as biologically inactive and detoxified forms (Cain et al., 2004). Changes in macroinvertebrate community composition stem from species-specific differences in sensitivity to metals that result in reduced abundance and ultimately local extinction of more sensitive macroinvertebrate species. Changes in macroinvertebrate species assemblages associated with increasing metal exposure appear to operate through the progressive selection against the least tolerant to the most tolerant taxa within a community (Cain et al., 2004). It is worth mentioning, however, that metal concentrations in the surface waters may not necessarily lead to lethality, growth or reproductive impairments. This is because uptake and toxicity of metals depend on their bioavailability that is influenced by the water quality variables among others (Van Sprang & Janssen, 2001). The bioavailable fraction of the metal has been shown to correspond to high mortality and reduced reproduction in fathead minnow (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) (Di Toro et al., 2001).

Data from recent studies strongly suggest that water chemistry plays a significant role in altering the potential toxicity of metals to aquatic organisms (Diamond et al., 1997; Di Toro et al., 2001). The fraction of a metal that is toxic to aquatic organisms depends on several variables including abundance of complexing ligands (organic and inorganic), water hardness, pH, dissolved organic carbon (DOC) (Newman & Jagoe, 1994; Erickson et al., 1996; Di Toro et al., 2001). Due to the dependence of metal speciation on the physiochemical properties of the solution, it is now widely recognized that local site water characteristics may significantly alter the toxicity of metals (U.S. EPA, 1994; U.S. EPA, 2002b). It is now widely accepted that metal –DOC interactions lead to the formation of relatively stable ligands that may be less toxic (Leppard, 1993; De Schamphelaere et al., 2004; Heijerick et al., 2003). The role of metal complexation in surface waters is critical because formation of organic and inorganic metal complexes renders a significant fraction of the total non-bioavailable metal (De Schamphelaere et al, 2004). Among other variables, water hardness and pH can significantly alter the bioavailability and therefore, toxicity of metals to the waterflea, *Ceriodaphnia dubia* Richard, 1894 (Schubauer-Berigan et al., 1993) and *Daphnia magna* Straus, 1820 (Campbell & Stokes, 1985).

The principal objectives of this study were threefold; (i) to characterize the distribution and concentrations of trace metals in surface water, (ii) to assess the impact of black shale-derived trace metals on the survival, growth and reproduction of the cladoceran, *Ceriodapnia dubia* and the fathead minnow, *Pimephales promelas* Rafinesque, 1820, and (iii) to assess the metal- water hardness, pH, and DOC interactions, and their impact on metal toxicity to *C. dubia* and *P. promelas*. Metal availability to aquatic organisms is a critical issue in the Little Red River, Arkansas, and its tributary black shale-draining streams due to the presence of the threatened/endangered Speckeled Pocketbook Mussel (*Lampsilis streckeri*) and the Yellowcheek Darter (*Etheostoma moorei*) (Wine et al., 2002; Winterringer et al., 2001). In this paper, we present results from water quality analyses together with toxicity bioassays using *C. dubia* and *P. promelas*.

Materials and Methods

Study sites description

The study area consisted of four headwater bedrock streams that drain into the Little Red River, Arkansas. The first three streams: Trace Creek, Begley Creek and Cove Creek flow over an extensive Mississippian Fayetteville Shale outcrop while the fourth stream is the Mill Creek that drains over the Mississippian Pitkin Limestone. Details on the study sites including drainage area characteristics are given in Ogendi et al. (2004b) and Ogendi et al. (2007a).

Water sampling

Water samples were collected using clean procedures (Shafer et al., 1997; Shiller 1997; Peucker-Ehrenbrink & Hannigan, 2000). All equipment used for sample collection, storage and analysis of trace metals were pre-cleaned using ultra-pure nitric acid (HNO_3; GFS Chemicals Inc.) and rinsed with copious amounts of 18.3 MegOhm water to ensure that they were trace-metal free. After rinsing, the sample containers were stored in double-bagged zip-lock polyethylene bags. Such cleaning and storage procedures ensure that there are no detectable metal contaminants in sample containers (Shafer et al., 1997; APHA, 1998). The water samples were collected in a flowing portion near the middle of each stream site. Samples for dissolved metals and DOC were filtered through 0.45 µm Gelman in-line filters, and acidified with 2 mL of ultra-pure HNO_3 to pH < 2, and stored in polypropylene bottles at 4 ^{o}C until the time of analysis. Ten liters of filtered and unacidified water samples were also collected for laboratory toxicity tests. All water quality variables known to affect the behavior of dissolved metals were measured. These parameters included dissolved oxygen, DOC, hardness, pH, and electric conductivity. Trace metals in filtrate (< 0.45 µm) are herein operationally defined as “dissolved”. We centered our attention on the dissolved metals because lethal and sublethal effects on aquatic organisms have been associated with this fraction (Di Toro et al., 2000). Other studies have also shown that dissolved metals represent exposure conditions to aquatic organisms (Prothro, 1993; U.S. EPA, 2002). This assumption allowed us to compare our findings to standard toxicity tests and make inferences concerning the potential impairments on the resident aquatic biota due to the elevated metals in the streams.

Water samples for dissolved organic carbon (DOC) were collected in 1-Liter glass jars (I-Chem Nalge Co. New Castle, DE, USA) equipped with TFE-backed septa. These jars were acid-washed and rinsed thoroughly with 18.3 MegOhm water. Prior to sampling, the Gelman in-line filters and the sample containers were flushed with aliquots of sample water. Water samples for DOC analysis were taken from the same stations that trace metal samples were collected. The water samples were analyzed for DOC within 48 hours of sample collection.

Trace metal analysis
Metal concentrations were determined by the Dynamic Reaction Cell Inductively Coupled Plasma Mass Spectrometry (DRCII ICP-MS; Elan PerkinElmer) following EPA 200.8 methodology (APHA, 1998). In brief, 15 mL of the 0.45 µm-filtered water sample was transferred into an autosampler vial into which an internal standard containing ^{6}Li, ^{75}Ge, ^{115}In, and ^{209}Bi was added. We then added 40 ug/L of ^{196}Au to the sample solutions to stabilize Hg. A standard calibration curve for all the analytes was established on standards prepared in a 2 % ultra pure nitric acid in a linear range from 1 ppb to 300 ppb. In order to monitor precision and accuracy, National Institute of Standards and Testing reference standard, NIST 1640, and procedural blanks were also analyzed. The trace metal concentrations were within 3% of the reported values for NIST 1640. Finally, the relative standard deviations (RSD) for sample trace metal concentrations were calculated and found to be < 5% for all analytes.

Water chemistry and culture of test organisms
The following water quality parameters were measured at the start and the end of the 7-day *C. dubia* and *P. promelas* chronic toxicity tests: temperature, dissolved oxygen, pH, and conductivity. These parameters were also determined on freshly prepared and 24-h-old solutions on a daily basis for 7 days. They were determined using the following meters and probes: pH was measured with a Accumet® Model 25 pH/ion meter (Fisher Scientific) or Orion pH meter Model 290A (Orion Research Inc., Boston, MA, USA); dissolved oxygen was measured with a YSI model 51B meter and probe (Yellow Springs Instrument Co. Inc. OH, USA). Temperature and conductivity were determined using YSI conductivity/DO/temperature meter Model 85 (Yellow Springs Instrument Co. Inc. OH, USA). Hardness and alkalinity were determined by titrating with 0.01 M ethylenediaminetetraacetic acid (EDTA) and 0.02 N sulfuric acid, respectively. Lastly, DOC analyses on laboratory and site water samples were done by UV-persulfate analysis (EPA 415.2) using a Tekmar-Dohrman Phoenix 8000™ TOC analyzer. All these water quality measurements remained within satisfactory limits (U.S. EPA, 2002a).

Ceriodaphnia dubia and *Pimephales promelas* were cultured in the Environmental Research Facility (ERF) at Arkansas State University following methods described in U.S. EPA (2002a). Briefly, the *C. dubia* and *Pimephales promelas* were maintained at 25±1 °C, with an illumination of approximately 700 lux and a 16:8-hr light:dark photoperiod. The physicochemical measurements of the water that was used for the test organisms' culture were within the specified ranges (U.S. EPA 2002a). Culture conditions for algae (*Selenastrum capricornatum* and *Chlorella spp.*) that were used as feed for the *C. dubia* were also within the specified limits (U.S. EPA, 2002). The algae were harvested in the exponential phase and stored at 4 °C.

Toxicity testing
The effect of site water was evaluated by conducting 7-day chronic static renewal toxicity tests following methods described in U.S. EPA (2002a). The test organisms included < 24 hour-old laboratory-cultured cladoceran, *Ceriodaphnia dubia* and fathead minnow, *Pimephales promelas*. Test organism survival, reproduction, and growth in site waters was evaluated and compared with that of synthetic laboratory moderately hard reconstituted water (control water; hardness: 99-100 mg/L and alkalinity: 64-66 mg/L). Toxicity endpoints for these chronic static renewal tests were survival and reproduction for *C. dubia,* and survival and growth for *P. promelas*. Toxicity tests were initiated within 24-hours of sample collection. For quality control reasons, only neonates from females that had produced more than 8 young in their third or subsequent broods were used. The adults were held individually for two weeks in 15-mL water that was renewed daily. Similarly, only healthy actively swimming < 24 h-old *P. promelas* larvae were used in fish toxicity tests. Ten less than 24 h old *P. promelas* larvae were randomly introduced into four replicate 250-ml test chambers per site, and their survival monitored for seven days.

The *C. dubia* were fed daily on 0.1 ml of laboratory-cultured YCT (Yeast, Cereal, and Trout chow) and an algal suspension (*Selenastrum capricornatum* – 75% and *Chlorella spp*.-25%). Daily renewal of water in test chambers was continued until 60% of the surviving control group *C. dubia* females produced three or more broods. The *C. dubia* tests met all criteria for organism performance in the control treatments including ≥ 80% survival and a mean of 15 neonates per surviving female (US EPA, 2002a). The fish were fed twice daily at 6-h intervals and the feed constituted 0.15 g of newly hatched brine shrimp nauplii (< 24 h old). The test was also terminated at day seven upon which the weights of the live fish was determined. Our fish toxicity tests met the test acceptability criteria required for organism performance in the control: ≥ 80% survival, and a mean weight of > 0.25 mg per individual in the control group (U.S.EPA, 2002a).

Statistical analyses
Prior to any statistical analyses, the toxicity response data from survival, reproduction, and growth endpoints of the two test organisms were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov Normality Test ($p > 0.15$) and Levene's Test for equal variances ($p > 0.05$), respectively. Those data that did not meet basic

normality and homogeneous variance assumptions were square-root-transformed before performing an analysis of variance (ANOVA) to test for differences among different site waters (treatment groups). Upon significant ANOVAs, each treatment group was compared to the control group using Dunnett's Test ($\alpha = 0.05$) (Dunnett, 1955), and then all pairwise comparisons were made among treatment groups using Tukey's Multiple Comparisons Test ($\alpha = 0.05$) (MINITAB 2000). The response variable in *C.dubia* tests was either the number of neonates produced per adult female or the percent survival per treatment group. On the other hand, for *P. promelas*, the mean weight of live fish and percent survival for each site provided a combined measure of the site water's effect on both mortality and growth as described in U.S. EPA (2002a). Tukey's Multiple Comparisons Test distinguished between the toxicity responses of the black shale-draining treatment groups from those of the limestone-draining treatment groups. Reproductive success was measured as the number of neonates produced per an adult *C.dubia*. Finally, multiple regression models were fitted with either *C. dubia* reproduction or *P. promelas* growth as the response variables, and metals, DOC, pH, and water hardness as the predictor variables. These multiple regressions enabled us to predict the amount of explained variation in reproduction or growth due to these predictors individually as well as due to interactions between predictors.

Results

Water chemistry

Significant variations in hardness, dissolved organic carbon (DOC), and conductivity were observed among site waters ($p < 0.05$). Except for the control water, the conductivity values for Trace, Begley, and Cove creeks were significantly higher than those for Mill creek throughout the sampling period (Table 1). Similarly, water hardness values for Trace Begley, and Cove creeks were significantly higher than those measured in Mill creek ($p < 0.05$; Table 1). The pH values for Trace creek samples were consistently lower than those measured in other sites throughout the sampling period with the highest values measured in Mill Creek. Significant differences ($p < 0.05$) were also observed in DOC among the site waters with the highest and lowest values occurring at Trace and Mill creeks, respectively (Table 1). However, no significant differences ($p < 0.05$) in dissolved oxygen were observed among sites throughout the sampling period. Potentially toxic trace metals such as copper, cadmium, mercury, and lead exceeded criterion continuous concentrations (Table 1). All metal concentrations except mercury and zinc were significantly higher ($p < 0.05$) in Trace and Begley creeks than Mill creek (Table 1). Concentrations of metals in Trace and Begley creeks were significantly higher than those measured in Cove Creek (Table 1).

Ceriodaphnia dubia toxicity responses

Ceriodaphnia dubia survival in the control treatments water was on average greater than 98% for the six tests (Fig. 1). Similarly, *C. dubia* reproduction in the control treatments was more than 24 neonates per adult surviving female. The month when sampling occurred ($F_{(5, 40)} = 0.67$; $p = 0.65$) and the source of water (i.e. sites) ($F_{(8, 40)} = 1.67$; $p = 0.137$) did not have significant impacts upon the survival of the *C. dubia* in the six tests that were conducted at different times. However, reproduction was significantly influenced by both the sampling occasion ($F_{(5, 526)} = 12.37$; $p = 0.001$) and the test water sources ($F_{(6, 526)} = 35.19$; $p = 0.001$). On average, neonate production was highest in water samples collected in June and lowest in September and November (Fig. 1).

On average, the number of neonates produced in the June test for every site was significantly greater than for the other tests ($p < 0.05$; Fig. 1). Neonate production in the June toxicity test was significantly different among sites ($F_{(8, 81)} = 6.52$; $p < 0.001$; 95% Confidence Interval (CI)) with organisms in Mill creek treatments producing larger broods than Trace, Begley, and Cove creeks (Fig. 1). Similar trends were observed in August ($F_{(8, 81)} = 10.04$; $p < 0.001$; Fig. 1), September ($F_{(8, 81)} = 8.43$; $p < 0.001$; Fig. 1), November ($F_{(8, 81)} = 4.11$; $p < 0.001$; Fig. 1), January ($F_{(8, 81)} = 4.86$; $p < 0.001$; Fig. 1), and May ($F_{(8, 81)} = 27.28$; $p < 0.001$; Fig. 1) with larger brood sizes in Mill creek than Trace, Begley, and Cove creeks (Fig. 1). Survival and neonate production were lowest in Trace creek for all the six tests (Fig. 1). The number of neonates in the control treatment was also significantly greater than that of Trace, Begley, and Cove creeks for all the six toxicity tests ($P < 0.05$; 95% CI). However, the number of young produced in the control treatment did not differ significantly from that observed in Mill creek treatments for all the six tests ($p > 0.05$; Fig. 1).

Pimephales promelas toxicity responses

Fathead minnow survival was significantly reduced among sites ($F_{(8, 207)} = 6.66$; $p < 0.001$) and among the six tests ($F_{(5, 210)} = 5.17$; $p < 0.001$). On average, percent survival in the control treatment was significantly higher than that Trace, Begley, and Cove creek treatments ($p \leq 0.05$). Survival in the control did not differ significantly from that observed in Mill creek treatment group ($p > 0.05$). Significant site differences were noted in survival for the June ($F_{(8, 27)} = 2.92$; $p = 0.05$), August ($F_{(8, 27)} = 4.02$; $p = 0.03$), September ($F_{(8, 27)} = 3.39$; $p = 0.05$), November ($F_{(8, 27)} = 2.77$; $p = 0.06$), January ($F_{(8, 27)} = 3.10$; $p = 0.04$), and May (F $(_{(8, 27)} = 3.17$; $p = 0.05$) toxicity runs. However, no statistical differences in survival of *P. promelas* were noted between Mill creek water and the control treatments ($p > 0.05$; Fig. 2).

Table 1. Criterion Continuous Concentrations (µg/L) of some potentially toxic metals, and physicochemical variables water samples collected from Trace, Begley, Cove, and Mill Creeks in north-central Arkansas, USA. Var. = Variables; DO = dissolved oxygen (mg/L); CD = conductivity (mS/cm); ALK = Alkalinity (g/L); HD = Hardness (g/L as $CaCO_3$); DOC = Dissolved organic carbon. Lowercase "*abcd*" notation: all values with same letter are not significantly different from each other ($p > 0.05$).

	Trace Cr.	Begley Cr.	Cove Cr.	Mill Cr.		Trace Cr.	Begley Cr.	Cove Cr.	Mill Cr.
Jun, 2003					*Nov, 2003*				
Cr	14.99a	9.43b	3.17c	0.49d	Cr	12.12a	7.65b	2.59c	0.45d
Ni	3.33a	3.58a	1.59b	1.49b	Ni	3.10a	3.24a	1.50b	1.40b
Cu	9.50a	14.00b	4.10c	4.13c	Cu	9.45a	12.50a	4.50b	4.10b
Zn	16.27a	14.54b	12.81b	17.90a	Zn	16.27a	14.64a	12.81b	17.95a
Se	4.74a	8.98b	7.14b	2.70c	Se	4.34a	8.18b	6.49b	2.50c
Cd	0.31a	0.28a	0.20b	0.08c	Cd	0.36a	0.23b	0.14c	0.09c
Hg	3.43a	2.35b	4.80c	6.11c	Hg	2.82a	1.92b	4.01c	0.11d
Pb	25.19a	8.10b	3.33c	5.02c	Pb	28.76a	9.20b	3.80c	5.70d
Aug, 2003					*Jan, 2004*				
Cr	1.68a	1.03b	0.32c	0.07d	Cr	3.03a	1.94b	0.65c	0.10d
Ni	1.88a	1.77a	0.84b	0.85b	Ni	2.09a	2.14a	0.95b	0.90b
Cu	3.77a	4.10a	1.30b	1.10b	Cu	2.63a	3.65a	1.32b	1.29b
Zn	8.71a	7.79a	6.26b	8.23a	Zn	8.43a	7.5a	6.06a	7.99a
Se	3.76a	7.08b	5.63b	2.13c	Se	4.24a	7.93b	6.33b	2.39c
Cd	0.26a	0.27a	0.14b	0.07c	Cd	0.23a	0.21a	0.09b	0.04c
Hg	0.78a	0.54a	1.14b	1.40b	Hg	1.01a	0.65b	1.41a	1.73c
Pb	6.14a	0.18b	0.10c	0.17b	Pb	6.06a	0.15b	0.08c	0.15b
Sept, 2003					*May, 2004*				
Cr	1.32a	0.88b	0.61c	0.66c	Cr	2.28a	1.49b	0.50c	0.09d
Ni	2.93a	3.10a	1.38b	1.33b	Ni	1.55a	1.60a	0.70b	0.70a
Cu	3.32a	3.90a	1.40b	1.26b	Cu	2.45a	3.23a	1.66b	1.27b
Zn	8.26a	7.39a	5.96b	7.83b	Zn	5.52a	4.93a	4.34b	6.06a
Se	3.38a	6.70b	5.04b	1.88c	Se	3.04a	5.74b	4.54b	1.70c
Cd	0.31a	0.29a	0.11b	0.08b	Cd	0.21a	0.25a	0.18a	0.07b
Hg	0.84a	0.60b	1.24c	1.48c	Hg	0.83a	0.58a	1.23b	1.52b
Pb	4.72a	0.15b	0.08c	0.09c	Pb	4.19a	0.11b	0.09b	0.15c
Other var.									
pH	5.15a	5.56a	7.16b	7.56b	ALK	0.32a	0.34a	0.31a	0.37a
DO	7.45a	6.87a	7.78a	6.89a	HD	0.17a	0.15a	0.13a	0.15a
CD	0.28a	0.29a	0.25a	0.12b	DOC	12.54a	14.61a	8.43b	4.59c

Growth of the fathead minnow in the various site waters differed significantly ($F_{(8, 27)} = 8.19$; $p < 0.001$). In the June test, fish in the control treatment group were significantly heavier than those of Trace, Begley, and Cove creeks ($p < 0.05$). Similar observations were made in September ($F_{(8, 27)} = 3.43$; $p = 0.01$), November ($F_{(8, 27)} = 2.30$; $p = 0.05$), and May ($F_{(8, 27)} = 7.77$; $p < 0.001$) tests with significant growth reductions in Trace, Begley, and Cove creeks compared to Mill creek treatments (Fig. 3). However, no site differences in growth were noted between Mill creek and the control group ($p > 0.05$; Fig. 3).

Metal-DOC, pH and hardness interactions

Multiple regression analyses with *C. dubia* reproduction and *P. promelas* growth as the response variables and dissolved metal, DOC, hardness and pH as the predictor variables yielded high p-values ($p > 0.10$) for the interaction terms. One-way ANOVA indicated that the predictor variables were important in explaining the variation in *C. dubia* ($F_{(8, 45)} = 26$; $p < 0.001$). A detailed examination of the t-values and the corresponding p-values from the regression models indicated that water chemistry variables, the dissolved copper, and sites explained 82.2% (R^2) of the variation in *C. dubia* reproduction (Table 2). Among the measured variables, sampling occasion did not significantly influence *C. dubia* reproduction (t-value = -0.63; $p = 0.53$). Mercury had a negative but insignificant impact on the *C. dubia*

The regression model indicated that sites, DOC, Cd, Cu, pH, and water hardness were significant factors in explaining the variation in reproduction (Table 2). One-way ANOVA indicated that the predictor variables were important in explaining the variation in *P. promelas* growth ($F_{(8, 45)} = 9.04$; $p < 0.01$). A closer examination of the t-values associated with the individual predictor variables showed that some of them had significant impacts on the fish growth, while others did not. Mercury, Cu, DOC, water hardness, sites, and sampling occasion had significant effects on fish growth (Table 2). The predictor variables in this regression model accounted for 61.6% of the observed variation in *P. promelas* growth.

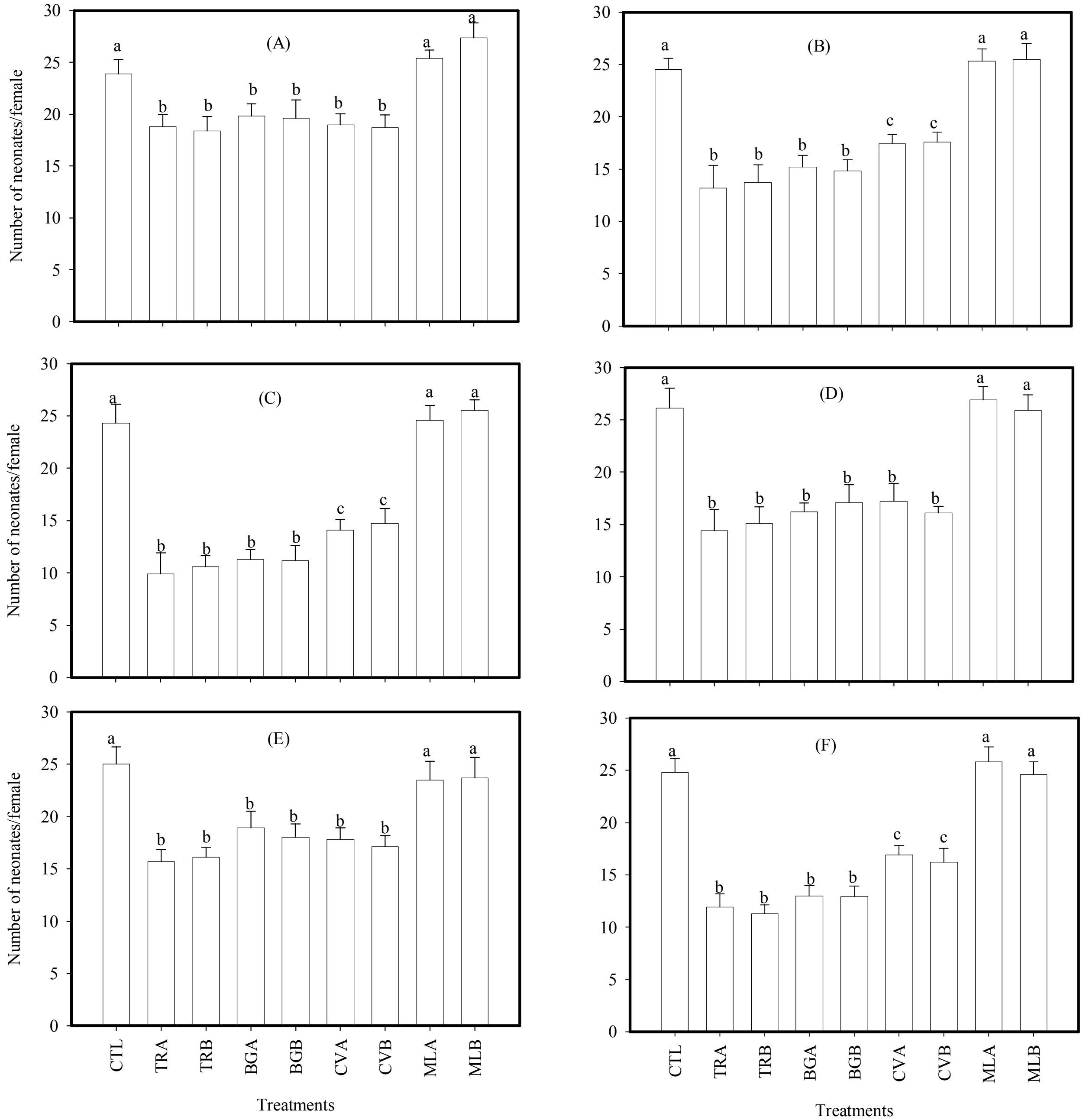

Fig. 1. Reproduction in waterflea, *Ceriodaphnia dubia* (mean ± standard error of neonates) in the control, reference and site waters. CTL = control treatment; treatments containing water samples collected from Trace Creek are coded TRA & TRB; those from Begley Creek as BGA & BGB; those from Cove Creek as CVA & CVB, and those from Mill Creek as MLA & MLB. The results are from laboratory experiments conducted in (a) June, 2003, (b) August, 2003, (c) September, 2003, (d) November, 2003, (e) January, 2004, and (f) May 2004. Lowercase "*abc*" notation on top of error bars: all sites labeled with same letter are not significantly different (p > 0.05). reproduction (t-value = -0.22; p = 0.83; Table 2).

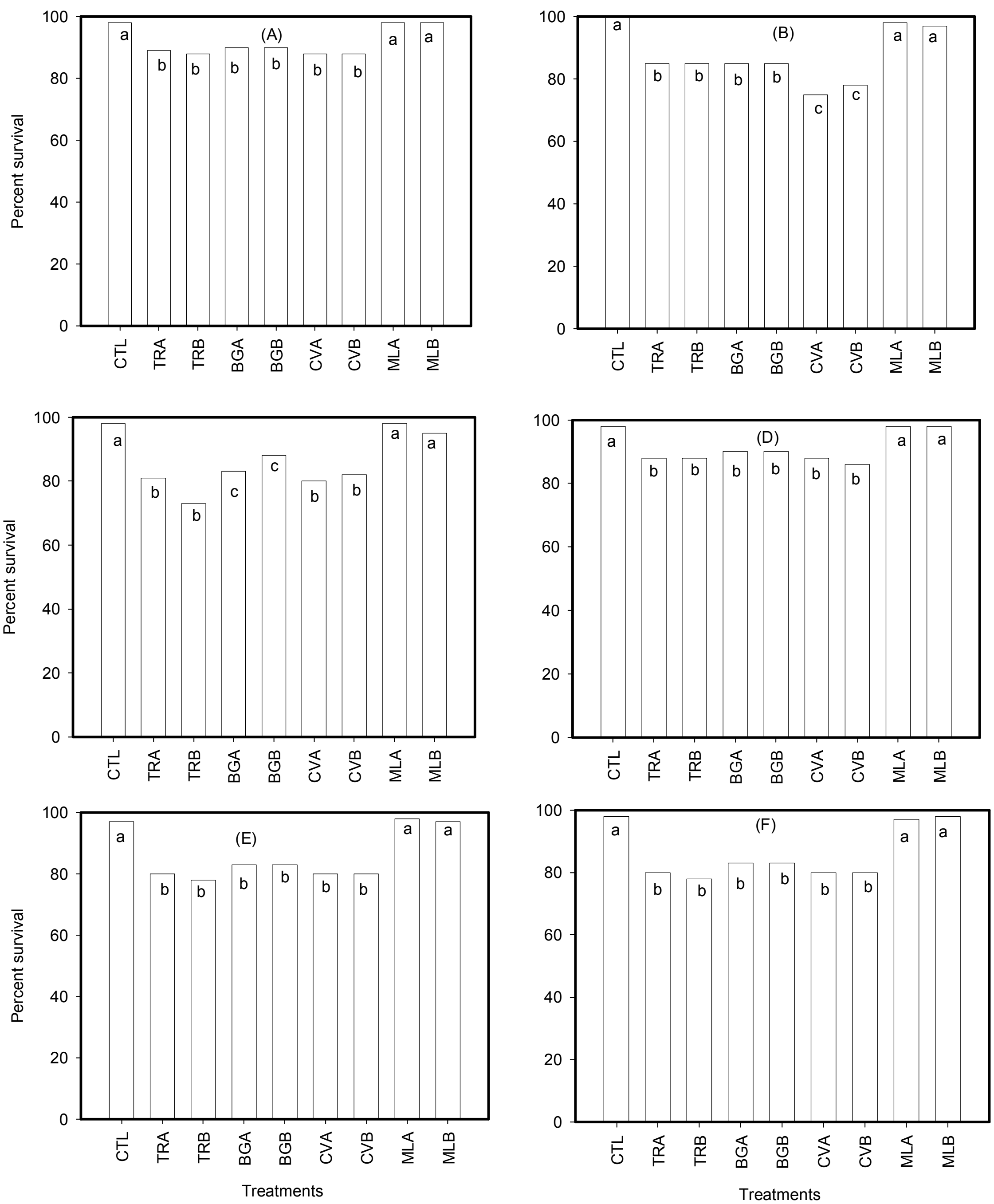

Fig. 2. Percent survival of fathead minnow, *Pimephales promelas* in the control, reference and site waters. See Figure 1 for the description of *X-axis* codes. The results are from laboratory experiments conducted in (a) June, 2003, (b) August, 2003, (c) September, 2003, (d) November, 2003, (e) January, 2004, and (f) May 2004. Lowercase "*abc*" notation in the bars: all sites labeled with same letter are not significantly different ($p > 0.05$).

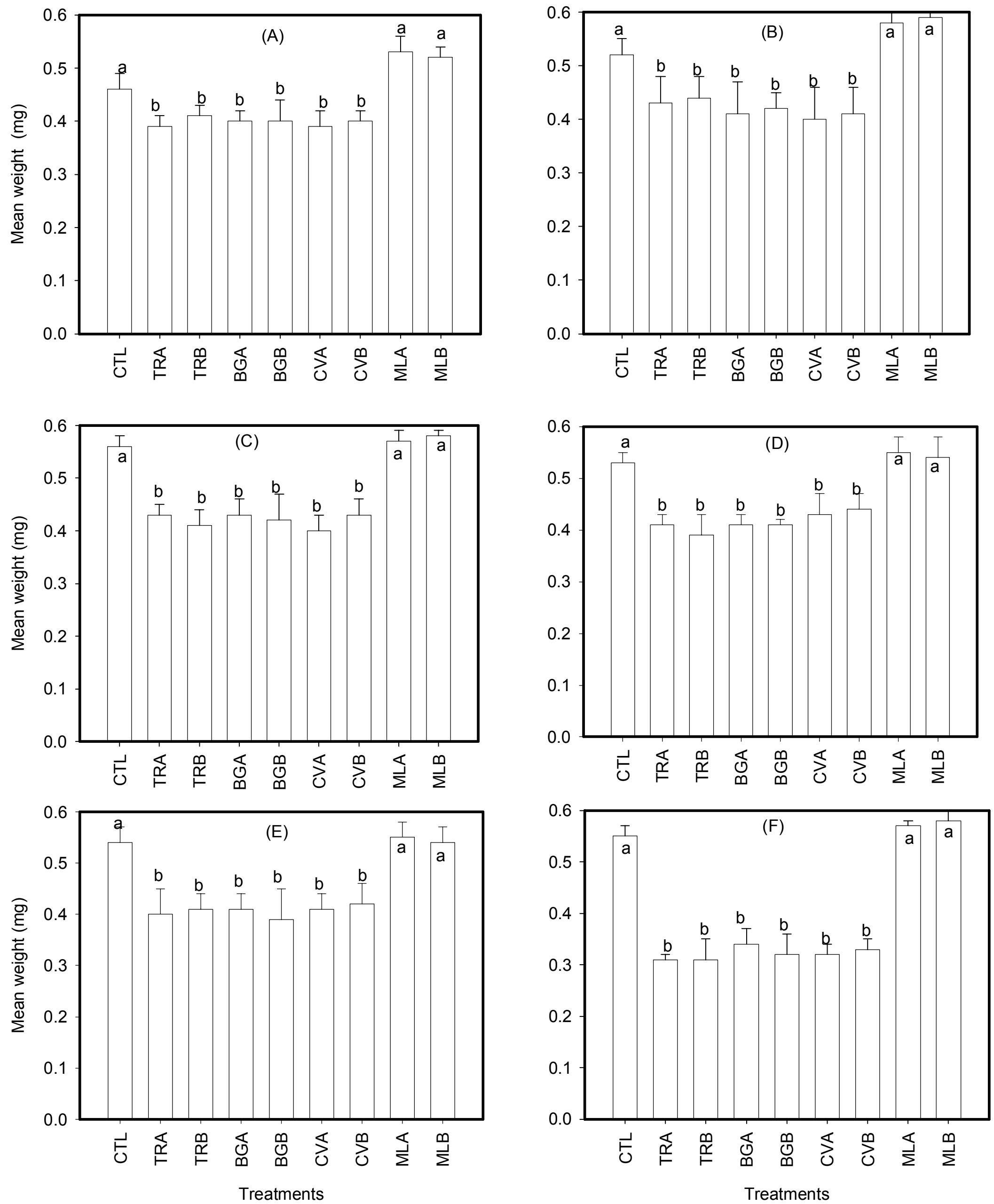

Fig. 3 Growth of fathead minnow, *Pimephales promelas* (mean ± standard error) in the control, reference and site waters. See Figure 1 for the description of *X-axis* codes. The results are from laboratory experiments conducted in (a) June, 2003, (b) August, 2003, (c) September, 2003, (d) November, 2003, (e) January, 2004, and (f) May 2004. Lowercase "*ab*" notation in the bars: all sites labeled with same letter are not significantly different (p > 0.05).

Discussion

The water samples from black shale draining streams can generally be described as moderately hard to very hard water with hardness values ranging from 120 to 200 mg/L $CaCO_3$/L (Table 1) while those from Mill creek can be classified as hard water (U.S. EPA, 1989). The high water hardness values in the limestone draining Mill Creek samples can be attributed to large amounts of calcium and magnesium ions (Ogendi et al., 2004b). This observation as expected due to the dominance of calcium carbonate rocks in the watersheds of Mill creek.

High hardness values in streams draining over black shales may also be attributed calcium carbonate cement in addition to a variety of metals that may substantially contribute to high water hardness. However, to what extent the soil mineralization or groundwater plays in hardness is unknown. The relatively high conductivity values observed in our study sites may be explained by the presence of cations and anions that get dissolved readily as water moves through soils and rocks in the watershed. The explanation for the low pH values observed in Trace and Begley creeks is unclear. However, some authors have attributed this to dissolution of pyrite (Tuttle et al. 2004). Low pH values have been encountered in surface waters flowing through black shales (Loukola et al., 1996; Tuttle et al., 2001; Tuttle & Breit, 2004). Oxidation of the sulfide mineral results in acidic, metal rich solutions that are transported to the streams by the rainwater. The geology of a watershed contributes substantially to the amount of DOC in streams (Shafer et al., 1997; Shiller et al., 1997; Ogendi et al. 2007b). The significantly high levels of dissolved organic carbon in streams draining black shales were attributed to the organic carbon in black shales. Studies have demonstrated that black shales are enriched in organic carbon, and therefore a possible explanation for high DOC values in Trace, Begley and Cove creeks (Petsch et al., 2000; Ogendi et al., 2004b, 2007a). Trace metal enrichment in Trace, Begley, and Cove creek surface waters can be attributed to weathering of black shales in their watersheds. The occurrence of elevated metal concentrations in black shale rocks, sediments, and soils has been demonstrated by Hannigan (1997), Lee and et al. (1998), Tuttle and Breit (2004), and Ogendi et al (2007a, 2007b).

Table 2. Multiple regression analyses for *Ceriodaphnia dubia* reproduction and *Pimephales promelas* weight (as response variables) vs. metal, DOC, pH, hardness, sites, and sampling occasion (as predictor variables) for water samples collected from Trace, Begley, Cove, and Mill Creeks in north-central Arkansas, USA.

	Ceriodaphnia dubia		*Pimephales promelas*	
Variable	T-value	*p-value*	T-value	*p-value*
Cadmium	-3.23	<0.01	-0.69	0.49
Mercury	-0.22	0.83	-2.57	0.01
Copper	-4.25	< 0.01	-2.25	0.03
DOC	2.12	< 0.05	2.37	0.02
pH	1.76	0.08	0.64	0.54
Hardness	5.89	< 0.01	2.43	0.01
Sites	-2.91	< 0.01	2.25	0.03
Sampling occasion	-0.63	0.53	-4.11	<0.01

Pimephales promelas and *Ceriodaphnia dubia* are generally considered appropriate surrogates for native species of biota, which form important links in aquatic food webs that include fish, frogs, waterfowls, and other higher trophic organisms (Viganò et al., 1996; Viganò, 2000). *Pimephales promelas* and *Ceriodaphnia dubia* have been used widely in surface water monitoring due to their sensitivity to pesticides, industrial chemicals, metals, (Knight & Waller, 1987; Anderson & Benke, 1994) and aquaculture effluents (Stephens & Farris, 2004). The data acceptability criteria for our toxicity tests were satisfied in all tests for both *C. dubia* and *P. promelas* (U.S. EPA, 2002). Despite the observed differences in both survival and growth of *P. promelas* among treatment groups, on average, except in a few cases, the endpoints were equal to or greater than the minimum 80% survival and 0.25 mg mean weight per individual proposed as minimum criteria for control group surviving individuals. The similarity of waterflea reproduction and fathead minnow growth responses in the control and Mill creek site waters can be attributed to the observed low metal concentrations particularly Cd, Cu, Hg and Pb. The impact of Mill creek site water on growth and reproduction of the two test organisms was minimal. The higher *C. dubia* reproduction in Mill creek compared to that of the control treatments may be due to absence of certain essential elements such as selenium in the moderately hard reconstituted water that was used in the control treatments. Bailey and et al. (2000) found that *C. dubia* reproduction increased by more than 10% when selenium was added to the test water. It may also have been due to the presence of symbiotic bacteria and additional algae in Mill Creek water.

In this study, the near neutral pH, high DOC, and water hardness in the black shale draining streams, account for the low observed lethal and sublethal effects of the test organisms. Toxicity of metals in freshwaters may be modified by various abiotic factors including: pH, dissolved organic carbon (DOC), and hardness (Schubauer-Berigan et al., 1993; Heijerick et al., 2003). Van Sprang and Janssen (2001), and Campbell and Stokes (1985) confirmed pH-dependency of Cd toxicity on *C. dubia*. They suggested that the low Cd toxicity may be explained by increased H^+ competition for binding sites or a change in the membrane potential. Pagenkopf (1983), and Di Toro et al. (2001) also concluded that Cu toxicity to fathead minnows and rainbow trout may be greatly reduced due to the competition of the metal with other cations such as Ca^{2+} and H^{+}. Van Sprang and Janssen (2001) linked the decline in cadmium toxicity with increasing pH to the formation of insoluble metal precipitates. Natural humic and fulvic acids have been found to be strong complexing agents that decrease the bioavailable fraction of trace metals (Allen & Hansen 1996, Hocket & Mount 1996, Kim et al. 1999).

Although DOC and pH determine which metal species are present in water, hardness ions compete effectively with free metal ions for binding surfaces on organisms (Erickson et al., 1996; Allen et al., 1998). Such binding sites include fish gill membranes and dissolved organic matter. The EC_{50} values for the chronic toxicity for *Daphnia magna* increased four-fold when hardness and DOC were increased from 35 to 270 (mg/L $CaCO_3$) and from 21 to 40 (mg/L DOC), respectively (Heijerick and Jansen 1998). De Schamphelaere et al. (2004) also found that overall, Cu toxicity was lower at higher DOC concentrations and higher pH levels. In the present study, water hardness was an important predictor in explaining the variations in *C. dubia* reproduction and *P. promelas* growth. It had a significant positive impact upon *C. dubia* reproduction ($t = 5.89$; $p < 0.01$) and *P. promelas* growth ($t = 2.43$; $p = 0.01$; Table 2). The surface waters of Trace, Begley, Cove, and Mill Creeks would be classified as moderately hard to very hard waters with hardness values ranging from 120 to 200 mg $CaCO_3$/L. Naddy et al (2002) noted that higher calcium: magnesium ratios (i.e. Ca: Mg > 1) can significantly alter the toxicity of copper. Since this ratio was significantly higher in surface waters of the Little Red River, greater reduction of copper toxicity was expected. The current findings have some similarities with those of Erickson et al. (1996) where high pH, DOC, and hardness significantly reduced copper toxicity to fathead minnows and waterfleas (Kim et al., 1999). Finally, we observed strong correlations between metals and DOC ($r^2 = 0.53 – 0.91$; Ogendi et al. 2007b) and thus greater metal-DOC complexation which may have rendered much of the Cu, Cd, and Hg biologically unavailable for uptake by the test organisms.

Conclusions

Ceriodaphnia dubia and *P. promelas* toxicity bioassays were able to differentiate varying levels of metal concentrations in the studied stream systems, with higher metal concentrations corresponding with reduced growth, decreased reproduction and higher mortality. Toxicity test results from this study were in agreement with the measured water quality variables. Our toxicity test findings also indicated that, whereas the concentrations of copper, cadmium and mercury in streams draining black shales can exceed the criterion continuous concentrations (CCC), their impact upon aquatic organisms may be minimal due other confounding factors. The negative impacts of potentially toxic metals upon waterflea and fathead minnow survival, growth, and reproduction were lower than expected due to high DOC and cations that competed with the metals for biotic ligands including the gill surfaces. Dissolved organic carbon, water-hardness cations as well as pH may have partly contributed to a decrease in the bioavailable fraction of the metals through formation of stable organic and inorganic complexes. Despite the presence of potentially toxic metals in levels exceeding the CCC proposed by National Surface Water Quality Criteria (USEPA, 2002b), aquatic organisms in streams draining through black shales may be thriving due to the toxicity-mitigating effects of water hardness, DOC and pH. Nevertheless, given the range of dynamic conditions in these streams, black shale-sourced metals may still be bioavailable to cause lethality, and growth and reproduction impairments to resident aquatic organisms including macroinvertebrates. To further understand the impacts of these naturally-derived metals, a study is underway investigating the spatial and temporal distribution, and metal body burdens of macroinvertebrates in the Little Red River tributaries.

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