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SALINITY AS A LIMITING FACTOR FOR BIOLOGICAL CONDITION IN MINING-INFLUENCED CENTRAL APPALACHIAN HEADWATER STREAMS[1]

Anthony J. Timpano, Stephen H. Schoenholtz, David J. Soucek, and Carl E. Zipper[2]

ABSTRACT: Recent studies have found that Appalachian coal mining causes increased surface water salinity, and that benthic macroinvertebrate communities in salinized mining-influenced streams differ from communities in streams draining unmined areas. Understanding the role of salinity in shaping these communities is challenging because such streams are often influenced by a variety of stressors in addition to salinity. We characterized associations of salinity with biotic condition while isolating salinity from other stressors through rigorous site selection. We used a multimetric index of biotic condition to characterize benthic macroinvertebrate communities in headwater streams in the Central Appalachian Ecoregion of Virginia across a gradient of sulfate-dominated salinity. We found strong negative seasonal correlations between biotic condition and three salinity measures (specific conductance, total dissolved solids, and SO_4^{2-} concentration). We found no evidence to suggest stressors other than salinity as significant influences on biotic condition in these streams. Our results confirm negative associations of salinity with benthic macroinvertebrate community condition, as observed in other studies. Thus, our findings demonstrate that elevated salinity is an important limiting factor for biological condition in Central Appalachian headwater streams.

(KEY TERMS: invertebrates; biotic integrity; environmental impacts; conductivity; coal mining.)

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INTRODUCTION

Benthic macroinvertebrate communities in Appalachian streams draining coal mines often differ from communities found in streams in the absence of mining influence (Green *et al.*, 2000; Paybins *et al.*, 2000; Pond, 2004; Hartman *et al.*, 2005; Merricks *et al.*, 2007; Pond *et al.*, 2008). Elevated levels of dissolved ions (i.e., salinization) originating from coal mines have been suggested as a primary aquatic life stressor in such streams (e.g., Green *et al.*, 2000; Pond, 2004; Pond *et al.*, 2008; Lindberg *et al.*, 2011; Cormier *et al.*, 2013). The United States (U.S.) Clean Water Act is intended to protect the capacity of streams to maintain biological integrity, and its enforcement in the Appalachian mining region relies heavily on benthic macroinvertebrates as indicators of overall biological condition (e.g., VDEQ, 2010a). Thus, aquatic life impacts from coal-mining-induced salinization have become a public policy concern (e.g., USEPA, 2011).

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[2]Graduate Student (Timpano), Department of Forest Resources and Environmental Conservation, Cheatham Hall Rm 210, 310 West Campus Dr.; Professor and Director (Schoenholtz), Virginia Water Resources Research Center, and Professor (Zipper), Department of Crop and Soil Environmental Sciences, Virginia Tech, Blacksburg, Virginia 24061; and Ecotoxicologist (Soucek), Illinois Natural History Survey, Champaign, Illinois 61820 (E-Mail/Timpano: atimpano@vt.edu).

Salinization of freshwaters and associated impacts are not unique to mining-influenced Appalachian headwater streams (Williams, 1987; Goetsch and Palmer, 1997; Kefford, 1998; Leland and Fend, 1998). However, in contrast to the above-referenced studies in which sodium and chloride are the dominant ions, Appalachian streams influenced by coal mining are most often dominated by dissolved anions SO_4^{2-} and HCO_3^-, with Ca^{2+} and Mg^{2+} as the most prominent cations by mass (Pond *et al.*, 2008; Timpano *et al.*, 2010; Lindberg *et al.*, 2011). Dissolved ions, at concentrations above background levels in Appalachian streams, have been shown to cause lethal and sublethal effects to a variety of freshwater invertebrates in laboratory toxicity tests (Mount *et al.*, 1997; Chapman *et al.*, 2000; Kennedy *et al.*, 2003, 2004; Soucek and Kennedy, 2005; Kunz *et al.*, 2013). Additional research has shown that ion toxicity to test organisms at any given concentration can vary with the type and combination of ions in solution (Goetsch and Palmer, 1997; Mount *et al.*, 1997; Kennedy *et al.*, 2005; Soucek and Kennedy, 2005). Although field studies have found altered aquatic communities in streams affected by coal mining, understanding the effects of salinity on those communities is challenging because such streams are often influenced by a variety of salinity-covariant stressors such as degraded habitat, sedimentation, metals (Howard *et al.*, 2001; Hartman *et al.*, 2005; Pond *et al.*, 2008), residential land uses (Merriam *et al.*, 2011), and, in areas with historical mining, acidic pH (Freund and Petty, 2007).

The objective of the research reported here was to characterize associations between salinity and biotic condition by effectively isolating salinity from other stressors through rigorous selection of study sites. In our view, this approach allows field-based determination of a salinity level that is limiting to the aquatic community, as opposed to a salinity level that is associated with a biological condition where nonsalinity stressors may be influential as well. Biotic condition was quantified using the Virginia Stream Condition Index (VASCI), a multimetric index of benthic macroinvertebrate community composition that is U.S. Environmental Protection Agency (USEPA) approved for enforcement of the Clean Water Act in Virginia's noncoastal streams (Burton and Gerritsen, 2003; VDEQ, 2010a).

METHODS

Site Selection

First- and second-order streams (Strahler, 1957) within the Virginia portion of Ecoregion 69 (Omernik, 1987) were selected such that all observable factors other than salinity were comparable to reference streams in the region (as described by USEPA, 2006). In selecting sampling reaches, we attempted to avoid influence from major upstream tributaries through examination of the National Hydrography Dataset (U.S. Geological Survey) and site reconnaissance to ensure our study reaches had no perennial or intermittent tributaries immediately upstream. We selected elevated salinity, or "test" sites, meeting all abiotic reference criteria (with the exception of specific conductance [SC]) used for Virginia Clean Water Act implementation studies (Burton and Gerritsen, 2003; VDEQ, 2006) (Table 1). Stream candidates were chosen by examining a variety of available water quality and land-use data using a geographic information system (ArcGIS; ESRI Inc., Redlands, California), augmented by consultation with mine operators, consultants, and regulators with specific knowledge of stream conditions within the study area, and by analysis of data concerning water quality, mine permits, and historical surface-mining site locations provided by Virginia Department of Mines, Minerals, and Energy.

More than 180 candidate sites were visited to assess suitability for study. Site reconnaissance allowed verification of current land uses and confirmation of reference-quality conditions, as per study design. Physicochemical water parameters, including pH and SC, were measured to ensure nonacidic conditions and that a gradient of salinity among test sites was achieved. Reference-quality habitat was assured by conducting qualitative visual estimates of habitat parameters using the high-gradient stream method as specified in USEPA's Rapid Bioassessment Protocols (RBP) (Barbour *et al.*, 1999). In addition, potential sources of nonpoint source pollution were

TABLE 1. Abiotic Criteria for Selection of Reference and Test Streams.

Parameter or Condition (units or range)	Selection Criterion
Dissolved oxygen (mg/l)	≥6.0
pH	≥6.0 and ≤9.0
Epifaunal substrate score (0-20)	≥11
Channel alteration score (0-20)	≥11
Sediment deposition score (0-20)	≥11
Bank disruptive pressure score (0-20)	≥11
Riparian vegetation zone width score, per bank (0-10)	≥6
Total RBP habitat score (0-200)	≥140
Residential land use immediately upstream	None

Note: From Burton and Gerritsen (2003); RBP, Rapid Bioassessment Protocols habitat, high-gradient streams (Barbour *et al.*, 1999).

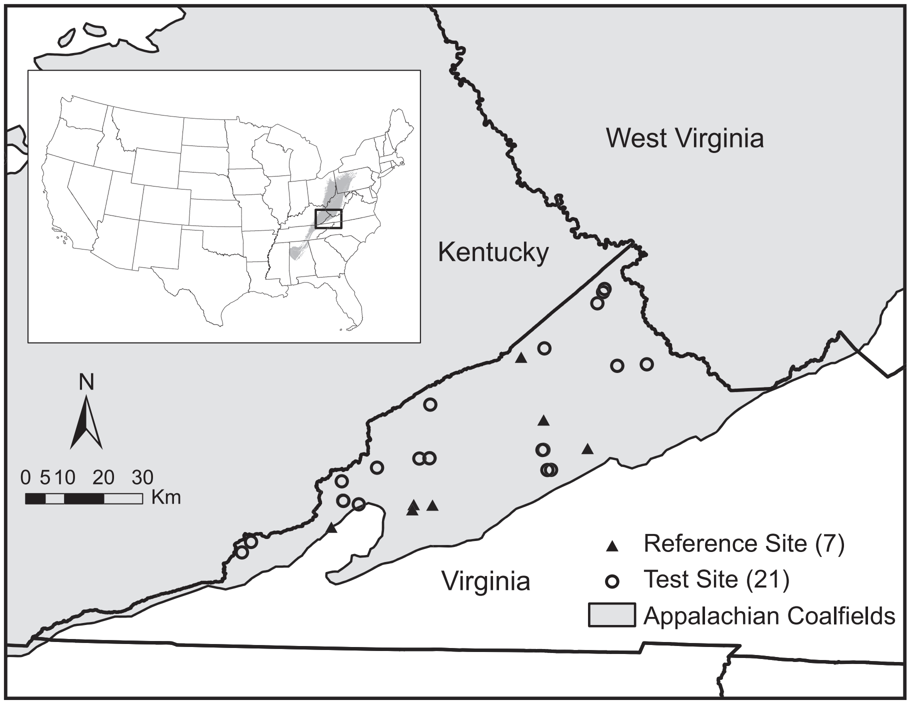

FIGURE 1. Map of Reference (n = 7) and Test (n = 21) Site Locations in the Appalachian Coalfield Region of Southwestern Virginia, U.S.

avoided, including upstream residential land use, road crossings, bridges, culverts, active logging, non-mining industrial operations or infrastructure (e.g., railbeds), and commercial activity. We selected 21 test sites and seven reference sites for study (Figure 1). Each salinized study site was an independent stream segment, with no other study sites upstream or downstream. Reference-site data were used to establish reference-quality habitat for ensuring test sites were comparable to reference sites in that respect. Reference sites represent minimally disturbed sites for the region of study (e.g., >50% of reference sites are in national forest).

Field Methods

At each study site, benthic macroinvertebrate and water quality samples were collected up to four times during the study period. Samples were collected during the Fall (September through November) of 2008 (n = 8) and 2009 (n = 25), and Spring (March through May) of 2009 (n = 20 sites) and 2010 (n = 28) benthic macroinvertebrate sampling index periods (VDEQ, 2008). Benthic macroinvertebrate collections followed the single-habitat (riffle-run) approach (VDEQ, 2008). Using a 0.3-m D-frame kicknet with 500-μm mesh, a single composite sample composed of six 1 × 0.3-m kicks was collected along a 100-m reach at each site, preserved in 95% ethanol, and returned to the laboratory for sorting and identification. Instream and riparian habitat quality was assessed during each sample collection using the same RBP method used for site selection.

Water temperature, dissolved oxygen (DO), SC, and pH were measured *in situ* with a calibrated handheld multiprobe meter (Hydrolab Quanta; Hach Hydromet, Loveland, Colorado). Single grab samples of streamwater were filtered using acid-rinsed cellulose ester filters with a nominal pore size of 0.45 μm and stored in acid-rinsed polypropylene bottles. Samples for trace elements analysis were preserved to pH < 2 with 1 + 1 concentrated ultrapure nitric acid. All samples were transported on ice and stored at 4°C until further analysis. Biological and water samples were collected concurrently at base flow.

Laboratory Methods

Processing of biological samples followed modified Virginia Department of Environmental Quality (VDEQ) biomonitoring protocols (VDEQ, 2008). Each sample was subsampled randomly to obtain a 110 (±10%) organism count following RBP methods (Barbour *et al.*, 1999). Benthic macroinvertebrates were identified to the family/lowest practicable taxonomic level using standard keys (Stewart *et al.*, 1993; Wiggins, 1996; Smith, 2001; Merritt *et al.*, 2008).

For water samples, an inductively coupled plasma-optical emission spectrometer (Varian Vista MPX ICP-OES w/ICP Expert software; Varian Instruments, Walnut Creek, California) was used to measure Ca^{2+}, Mg^{2+}, K^+, Na^+, and dissolved Al, Cu, Fe, Mn, Se, and Zn (APHA, 2005). An ion chromatograph (Dionex DX500; Dionex Corp., Sunnyvale, California) was used to measure Cl^- and SO_4^{2-} (APHA, 2005); total dissolved solids (TDS) was measured by drying of known volumes at 180°C (APHA, 2005), with modifications (0.45-μm filter, field filtration); total alkalinity was measured for an aliquot of filtered sample by titration with standard acid (APHA, 2005) using a potentiometric auto-titrator (TitraLab 865; Radiometer Analytical, Lyon, France); and HCO_3^- was calculated from alkalinity and pH measurements (APHA, 2005). Water chemistry data were examined to determine if selected trace elements were present at levels exceeding criteria continuous concentrations (CCC) (USEPA, 2012; ILEPA, 2001).

Data Analysis

We quantified biological condition using the VASCI, which uses family-level benthic macroinvertebrate taxonomic data to score streams from 0 to 100 relative to a reference condition with 100 being most comparable to reference. For aquatic-life designated-use assessment in Virginia, streams with VASCI scores < 60 are considered biologically impaired (VDEQ, 2010a). Taxonomic data were entered into the VDEQ Ecological Data Application System relational database (VDEQ, 2010b) from which VASCI scores were calculated using noncoastal statewide reference values for each metric (Burton and Gerritsen, 2003).

For each trace element, we calculated four method detection limits (MDLs), one for each batch analysis of samples, which were used for determination of analyte detection. Measurements less than batch MDL were reported as half batch MDL.

Water quality, habitat, and biotic metrics were compared between test and reference sites and between seasons using Wilcoxon's rank-sum test. Correlations among water quality parameters, biotic metrics, and VASCI scores were analyzed by Spearman rank correlation. We elected to model VASCI score as a function of SC, TDS, or SO_4^{2-} because those parameters are frequently identified as candidate stressors in the literature on salinization of Appalachian coalfield streams (e.g., Pond *et al.*, 2008; Lindberg *et al.*, 2011). Linear regression with mixed effects was used to construct models for Fall and Spring data, with VASCI score as dependent variable, salinity measure as fixed effect, and study site as random effect. Separate seasonal models were constructed because initial combined models indicated a significant seasonal effect. Site was treated as a random effect as a means of avoiding pseudoreplication from repeated samples at each site. Because we sampled benthic macroinvertebrates multiple times at each site, samples from a site are correlated with one another, thus introducing pseudoreplication from lack of statistical independence of samples (Hurlbert, 1984). Treating site as a random effect in regression models accounts for such correlation among repeated measures from the same site (Jiang, 2007). Each model was then used to estimate a biological effect concentration for Fall and Spring, which we defined as the salinity associated with the threshold of biotic stress (VASCI < 60). Seasonal biological effect concentrations were compared for differences using overlap analysis of 84% confidence intervals, which provides an approximation of a hypothesis test at the 0.05 level for data with equal variance (Payton *et al.*, 2003). All statistical analyses were conducted using R 2.15 (R Core Team, Vienna, Austria) with test level of $\alpha = 0.05$, unless noted otherwise.

RESULTS

VASCI Response

Mean (±SE) VASCI scores were significantly different between reference (78.3 ± 2.6) and test (58.2 ± 1.9) sites ($p < 0.0001$), with five of the eight VASCI component metrics significantly correlated with salinity (Table 2). Test-site VASCI scores were negatively correlated most strongly with TDS, SC, Ca^{2+}, and SO_4^{2-} (Table 3). Pairwise correlations among these four water quality parameters were strongly significant, with Spearman's $\rho > 0.90$ for each pair. Increasingly, weaker significant correlations were observed between VASCI and Mg^{2+}, K^+, HCO_3^-, and Na^+, respectively. Chloride was not significantly correlated with VASCI score. Regression models were significant for salinity measures (SC model: $p < 0.0001$, TDS model: $p < 0.0001$, SO_4^{2-} model: $p < 0.0001$), and season (SC model: $p = 0.0034$, TDS model: $p = 0.0061$, SO_4^{2-} model: $p = 0.0005$), with 77-89% of the variance in VASCI response explained by the models (Figure 2). Regressions satisfied error assumptions for statistical validity (linearity, independence, normality, and constant variance). We found that SC of 560 and 903 μS/cm corresponded to the threshold of benthic macroinvertebrate community stress (VASCI < 60) under Fall

TABLE 2. Virginia Stream Condition Index (VASCI) Component Metrics and Scores, and VASCI-Salinity Spearman Correlations.

			Spearman's ρ (n = 81)		
Metric	**Reference (n = 18)**	**Test (n = 63)**	**SC**	**TDS**	**SO_4^{2-}**
Total Taxa Richness	17.22 ± 0.6 (13-20)	12.81 ± 0.4* (5-22)	−0.64*	−0.65*	−0.58*
EPT Taxa Richness	12.28 ± 0.5 (8-16)	8.11 ± 0.4* (2-16)	−0.70*	−0.70*	−0.65*
% Ephemeroptera	20.97 ± 2.9 (1.98-41.28)	11.22 ± 1.4* (0-43.00)	−0.49*	−0.51*	−0.45*
% Plecoptera and Trichoptera less Hydropsychidae	38.04 ± 3.2 (18.02-71.29)	57.57 ± 2.1* (16.5-88.24)	−0.01	0.01	−0.03
% Scrapers	14.04 ± 1.8 (0.99-25.69)	4.62 ± 0.6* (0-18.69)	−0.51*	−0.52*	−0.55*
% Chironomidae	17.80 ± 2.6 (0-40.74)	7.52 ± 0.7* (0-23.93)	0.08	0.06	0.05
% Two Dominant Taxa	46.18 ± 1.9 (32.74-58.33)	64.56 ± 1.8* (38.6-96.08)	0.40*	0.42*	0.30*
Modified Family (Hilsenhoff) Biotic Index	3.40 ± 0.1 (2.25-4.29)	2.59 ± 0.1* (0.99-5.05)	0.16	0.16	0.16
VASCI Score	78.3 ± 2.6 (60.9-85.0)	58.2 ± 1.9* (42.8-78.2)	−0.63*	−0.64*	−0.58*

Notes: TDS, total dissolved solids; SC, specific conductance. Top value is mean ± standard error. Bottom value is range.
*Significantly different from reference sites or significant correlation (p < 0.05).

TABLE 3. Spearman Correlations for Test-Site VASCI Scores and Salinity Measures (n = 81).

	VASCI	TDS	SC	Ca^{2+}	SO_4^{2-}	Mg^{2+}	K^+	HCO_3^-	Na^+
TDS	−0.64								
SC	−0.63	0.99							
Ca^{2+}	−0.61	0.91	0.90						
SO_4^{2-}	−0.58	0.92	0.91	0.94					
Mg^{2+}	−0.55	0.87	0.85	0.95	0.94				
K^+	−0.54	0.90	0.89	0.82	0.83	0.81			
HCO_3^-	−0.42	0.64	0.67	0.45	0.38	0.35	0.63		
Na^+	−0.35	0.34	0.37				0.31	0.67	
Cl^-								0.30	0.58

Notes: VASCI, Virginia Stream Condition Index; TDS, total dissolved solids; SC, specific conductance.
All values shown are statistically significant (p < 0.05).

and Spring models, respectively (Table 4). Biological effect concentrations were significantly different between Fall and Spring seasons for all salinity measures (Table 4).

Isolating Salinity Effect

Analyses provided no evidence that measured potential stressors other than salinity significantly influenced VASCI scores. Individual habitat parameter values met reference criteria at the time of site selection and during the study at all sites, with two exceptions. Sediment deposition scored nine once at one site and bank stability scored 10 once at another site. Both observations were made at the end of the study (Spring 2010). Although mean (±1 SE) total habitat scores were significantly different between reference (179.7 ± 2.0) and test (168.8 ± 0.9) sites (p < 0.0001), and all individual habitat parameters except for Flow Regime (p = 0.3048), Channel Alteration (p = 0.4596), and Riffle Frequency (p = 0.2131) were significantly different between site types (Table 5), there were no significant correlations between individual habitat parameters and VASCI score (data not shown). In addition, all individual test-site mean total habitat scores were >85% of the mean reference-site habitat score, indicating that habitat for each of the 21 test streams was comparable to reference (Barbour *et al.*, 1999). All habitat scores were greater than the reference-site selection criteria used for Clean Water Act implementation studies in Virginia (Table 1).

Water Chemistry

Among measured physicochemical water quality parameters, mean (±1 SE) test-site pH (7.73 ± 0.1) was significantly different (p < 0.0001) from reference-site pH (7.07 ± 0.1) (Table 6), but there was no

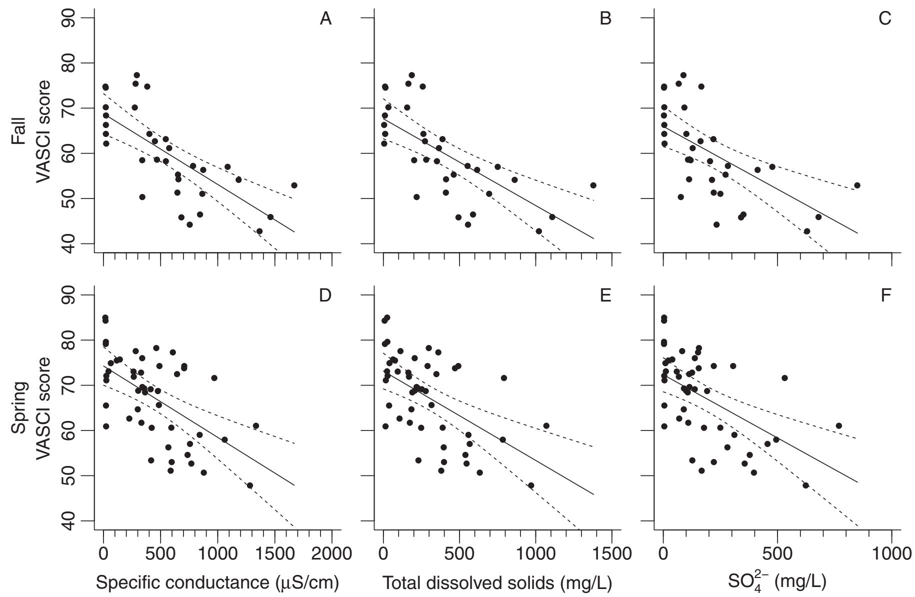

FIGURE 2. Scatterplots of Virginia Stream Condition Index (VASCI) Score *vs.* Salinity Measure by Season (Fall, n = 33: A-C; Spring, n = 48: D-F). With fitted mixed model regression lines (solid lines) and 95% confidence intervals (dashed lines). Note the consistent negative association between salinity and VASCI score, as well as the high variability of each relationship.

TABLE 4. Estimated Biological Effect Concentrations.

Salinity Measure	Season	Biological Effect Concentration	95% CI	R^2
SC (μS/cm)	Fall (n = 33)	560*	368-746	0.79
	Spring (n = 48)	903	700-1,351	0.78
TDS (mg/l)	Fall (n = 33)	398*	230-560	0.84
	Spring (n = 48)	665	500-1,043	0.78
SO_4^{2-} (mg/l)	Fall (n = 33)	214*	80-342	0.89
	Spring (n = 48)	441	321-727	0.77

Notes: CI, confidence interval; SC, specific conductance; TDS, total dissolved solids; R^2, coefficient of determination for mixed model.
*Significantly different from Spring model (p < 0.05).

significant correlation between test-site pH and VASCI score (p = 0.8864) and no pH measurements were outside the acceptable range of 6-9 for reference sites (Table 6). Water temperature (p = 0.2033) and DO (p = 0.4651) were not significantly different between reference and test sites, and no DO measurements were less than the minimum criterion of 6 mg/l for reference sites (Table 6).

Trace element concentrations were less than batch MDL in 65% of test-site samples. No test-site measurements exceeded USEPA CCC for Al, Cu (hardness-adjusted), or Fe. At test sites, only 2 of 63 samples exceeded the USEPA CCC for Zn (hardness-adjusted) and 9 of 63 samples exceeded USEPA CCC for Se. There is no USEPA aquatic-life water quality standard for Mn. Although Mn was significantly different between site types (p = 0.0268), all values reported here were at least one order of magnitude lower than

TABLE 5. Rapid Bioassessment Protocols Habitat Parameters.

Habitat Parameter	Reference (n = 18)	Test (n = 63)
Substrate/cover	18.7 ± 0.3 (15-20)	17.4 ± 0.1* (15-20)
Embeddedness	15.9 ± 0.4 (12-18)	14.2 ± 0.2* (11-17)
Velocity/depth regime	16.8 ± 0.6 (10-20)	15.6 ± 0.2* (10-20)
Sediment deposition	14.4 ± 0.4 (12-17)	12.6 ± 0.1* (9-14)
Channel flow status	18.6 ± 0.4 (14-20)	18.1 ± 0.2 (15-20)
Channel alteration	20 ± 0 (20-20)	19.9 ± 0 (17-20)
Riffle frequency	18.6 ± 0.3 (16-20)	18.2 ± 0.2 (16-20)
Bank stability	17.6 ± 0.4 (13-20)	15.6 ± 0.3* (10-20)
Vegetative protection	19.2 ± 0.3 (16-20)	18.4 ± 0.2* (14-20)
Riparian vegetative zone width	19.9 ± 0.1 (19-20)	18.9 ± 0.2* (15-20)
Total habitat score	179.7 ± 2 (162-190)	168.8 ± 0.9* (152-183)

Notes: Top value is mean ± standard error. Bottom value is range.
*Significantly different from reference sites (p < 0.05).

the proposed Illinois EPA aquatic-life CCC (Mn [dissolved] criterion = $0.9812(e^{4.0635+(0.7467*\ln(\text{hardness}))})$; B. Koch, Illinois EPA, personal communication), which is the only candidate aquatic-life criterion for Mn that we were able to identify. There were no significant correlations between Se, Zn, or Mn concentration and VASCI score (data not shown).

TABLE 6. Water Chemistry.

Parameter	Reference (n = 18)	Test (n = 63)
Temperature (°C)	11.09 ± 0.9 (1.74-15.03)	12.17 ± 0.4 (2.53-17.54)
pH	7.07 ± 0.1 (6.11-7.8)	7.73 ± 0.1 (6.57-8.49)*
Dissolved oxygen (mg/l)	9.54 ± 0.2 (7.69-11.81)	9.40 ± 0.1 (7.81-12.25)
Specific conductance (μS/cm)	30 ± 5.7 (16-116)	620 ± 42 (143-1,670)*
Total dissolved solids (mg/l)	21.89 ± 4.1 (5-76)	424.2 ± 35.1 (63.4-1,378.2)*
Total hardness (mg/l as $CaCO_3$)	9.79 ± 1.6 (2.87-32.04)	315.56 ± 27.4 (57.33-1,118.01)*
Ca^{2+} (mg/l)	2.45 ± 0.6 (0.38-11.98)	63.94 ± 5.1 (13.38-183.88)*
Cl^- (mg/l)	1.36 ± 0.3 (0.42-5.48)	3.68 ± 0.4 (0.31-15.1)*
HCO_3^- (mg/l)	10.30 ± 2.3 (0.72-44.1)	122.8 ± 8.4 (5.08-301.72)*
K^+ (mg/l)	0.61 ± 0.1 (0.33-1.38)	3.34 ± 0.2 (1.61-7.58)*
Mg^{2+} (mg/l)	0.9 ± 0.1 (0.47-2.6)	37.98 ± 3.7 (4.58-160.56)*
Na^+ (mg/l)	1.16 ± 0.2 (0.44-3.69)	25.90 ± 3.5 (0.81-135.95)*
SO_4^{2-} (mg/l)	5.67 ± 1.1 (2.76-22.14)	242.34 ± 23.5 (39.39-848.96)*
Trace elements		
Al (μg/l)	8.8 (1.4-41.9)	9.9 (1.4-50.5)
Cu (μg/l)	8.9 (4.4-11.4)	8.9 (4.4-18)
Fe (μg/l)	19.7 (11.1-319.9)	32.5 (11.1-410.9)
Mn (μg/l)	7.8 (0.8-15.2)	7.8 (0.8-787.9)*
Se (μg/l)	8.5 (2.5-18.4)	8.5 (2.5-28.3)
Zn (μg/l)	8.0 (3.7-18.7)	8.0 (3.7-116.0)

Notes: For trace elements, first value is median, followed by range. For others, first value is mean ± standard error, followed by range.
*Significantly different from reference sites ($p < 0.05$).

Test-site ion composition was dominated on a mass basis by SO_4^{2-} (46%), followed by HCO_3^- (27%), Ca^{2+} (13%), Mg^{2+} (7%), and Na^+ (6%) (Table 6). Potassium and Cl^- each comprised approximately 1% by mass of the test-site ion matrix. Reference sites were dominated by HCO_3^- (43%), followed by SO_4^{2-} (26%), Ca^{2+} (11%), Cl^- (7%), Na^+ (6%), Mg^{2+} (5%), and K^+ (3%) (Table 6). Spring and Fall relative ion proportions were not significantly different for either site type (data not shown).

DISCUSSION

Ion Matrix

Test-site water samples were dominated by SO_4^{2-}, HCO_3^-, Ca^{2+}, and Mg^{2+}, which is typical of alkaline mine drainage in the Appalachian region (Pond *et al.*, 2008; Lindberg *et al.*, 2011; Cormier *et al.*, 2013). Because the ion matrix of water influences ion toxicity (Mount *et al.*, 1997; Soucek and Kennedy, 2005), the results presented here are specific to the ion signature of the waters studied.

Biology-Salinity Relationships

In our study streams, biological condition was strongly and negatively associated with all three measures of salinity (SC, TDS, and SO_4^{2-}). This finding is consistent with results of other studies (Green *et al.*, 2000; Pond, 2004; Pond *et al.*, 2008; Gerritsen *et al.*, 2010; Bernhardt *et al.*, 2012). In our study streams, SC, TDS, and SO_4^{2-} concentrations were all highly correlated with one another (Table 3), suggesting that they could be used as surrogate measures within a salinity matrix, where SO_4^{2-} is the dominant anion and Ca^{2+} is the dominant cation. For this reason, we focus discussion on SC for the sake of brevity.

We found that for SC, our Spring biological effect concentration of 903 μS/cm is higher than those estimated by other investigators using Spring-Summer data from salinized Central Appalachian headwater streams. Effect concentrations in those studies were 180-300 μS/cm (Gerritsen *et al.*, 2010), 308 μS/cm (Bernhardt *et al.*, 2012), 426 μS/cm (Green *et al.*, 2000), 500 μS/cm (Pond *et al.*, 2008), and 501 μS/cm (Freund and Petty, 2007). This difference could be attributed to several factors. (1) Our biotic condition index differed from those used in the cited studies. With a different suite of constituent community metrics, we would not expect the VASCI to respond to salinity in a manner identical to the biotic indices used in those studies. (2) Our linear mixed modeling approach differs from the other studies, which used simple linear regression (Green *et al.*, 2000; Freund and Petty, 2007), generalized additive models (Bernhardt *et al.*, 2012), change-point analysis (Gerritsen *et al.*, 2010), conditional probability analysis (Gerritsen *et al.*, 2010), or no modeling (Pond *et al.*, 2008). Determination of biological effect concentrations for SC depends on how that relationship is quantified, and the range of values reported in the cited studies, despite using similar biotic indices, demonstrates the influence modeling framework can have on such determinations. (3) Biological sampling periods were not identical across studies. Our biological and chemical data for Spring were collected from March to May. Three of the cited studies used biological data from Spring samples (March-May), one study used data that could have been collected at any time from April to August (Bernhardt *et al.*, 2012), and one study used data that could have been collected at any time from April to October (Gerritsen *et al.*, 2010). Time of sampling could affect composition of the sampled assemblage, resulting in different biotic index scores (Šporka *et al.*, 2006). (4) Chemical sampling periods and model SC parameters differed across the cited studies as well. In our analysis and in most cited studies, SC was measured concurrently with biological sampling and used as-measured for modeling, but Freund and Petty (2007) used the mean of 12 monthly SC samples collected prior to biological sampling, and Green *et al.* (2000) used the median of five monthly SC samples collected prior to biological sampling. Timing of SC measurement and method of quantification could influence the resultant biological effect concentration. Such variation in sampling and modeling approaches makes direct comparisons among studies difficult. (5) Ion matrix differences could cause the SC value associated with biotic stress to vary. This explanation is unlikely, as our matrix is typical of alkaline mine drainage in the ecoregion (Pond *et al.*, 2008; Lindberg *et al.*, 2011; Cormier *et al.*, 2013). (6) Differences in habitat quality among studies could explain differences in biological effect concentrations for SC. Our rigorous site selection effort yielded test streams with good habitat quality that was comparable to reference stream habitat. Although the RBP approach for habitat assessment is a coarse measure, as it is simply a visual estimate, the high comparability of our test-site habitat to reference provides reasonable assurance that habitat quality was not a significant contributor to the biotic index scores we observed. Each of the cited studies controlled for habitat quality by ensuring that sites with poor habitat quality were not used in model development, but it is not clear whether test-site habitat scores in those studies were similarly comparable to their respective reference sites as we observed for our sites. Therefore, we can only posit that differences between reference and test-site habitat quality could explain some of the difference in salinity threshold values, but only if such habitat disparity was significant.

Our observed response of VASCI to salinity appears to be driven by taxonomic richness, as Total Taxa Richness and EPT Taxa Richness exhibited the strongest correlations with salinity (Table 2). Assemblage evenness is sensitive as well, as % Two Dominant Taxa is significantly and positively correlated with salinity (Table 2). Metrics that include richness or relative abundance of mayflies (i.e., Total Taxa Richness, EPT Taxa Richness, % Ephemeroptera, % Scrapers) are also significantly and negatively correlated with salinity (Table 2). The latter finding is consistent with observations by others (Pond, 2004, 2010) that mayflies are the benthic macroinvertebrate group most sensitive to elevated salinity in headwater streams of the Appalachian region. However, three other VASCI component metrics — % Plecoptera and Trichoptera less Hydropsychidae, % Chironomidae, and Modified Family (Hilsenhoff) Biotic Index — were not correlated significantly with salinity. These results suggest that salinity effects vary among taxa in the benthic macroinvertebrate community, manifesting with loss of specific salt-sensitive taxa (e.g., mayflies at the salinity levels observed in our study). Further investigation of community response independent of a multimetric index may yield enhanced understanding of salinity effects in these systems.

Seasonality

Analysis of site-wise paired VASCI scores from the final two seasons of data (the period with the greatest number of data pairs) indicated significantly higher VASCI scores in Spring 2010 than in Fall 2009 for test sites ($n = 21$, $p = 0.0051$; data not shown). In addition to biological variation, we also observed seasonal differences in salinity at test sites. All salinity measures were higher in Fall 2009 than in Spring 2010 (SC: $n = 21$, $p = 0.0003$; TDS: $n = 21$, $p < 0.0001$; SO_4^{2-}: $n = 20$, $p = 0.0005$; data not shown). Comparisons of regression models revealed similar seasonal differences. Nonoverlapping 84% confidence intervals for biological effect concentrations of all salinity measures indicated significant differences in seasonal thresholds (Table 4). These findings indicate that salinity-VASCI associations vary with season, suggesting that a given salinity level may not be associated with a single VASCI score at all times of the year. This underscores the importance of considering sample timing when conducting bioassessments and setting management goals.

Variability

Associations between salinity and biological condition were found to be strong, with approximately 77-89% of the total variance explained by our mixed models. However, confidence intervals for biological effect concentrations indicated variability in the associations observed. Variability sources other than the model parameters were not identified by our study, but several candidates can be suggested. Precision of the VASCI as developed is estimated at ±7.9 points (Burton and Gerritsen, 2003), which corresponds to approximately 10.1 and 13.6% of reference and test-site means, respectively. Unobserved nonsalinity stressors may be influencing VASCI in addition to salinity. As an example, seasonal thermal regime is an important factor affecting aquatic insect communities (Vannote and Sweeney, 1980). Mean stream temperatures at our test sites were nominally higher than at reference sites, but we did not collect data for summer maximum temperatures. Other researchers have found differences in hydrologic responses of mined and unmined watersheds, with mined watersheds producing higher peak flows (Bonta *et al.*, 1997; Negley and Eshleman, 2006); we did not consider hydrologic response flows as a site-selection criterion. Fine-resolution, quantitative habitat measures may explain some of the variability observed in our associations, but such measurement was beyond the scope of our study. Although not considered a stressor per se, an unknown factor is the temporal pattern of salinity exposure, which could influence biotic response (USEPA, 2000). By measuring water quality only twice per year, we could not quantify the magnitude and duration of salt exposure, nor could we determine if the benthic macroinvertebrate community was exposed to transient spikes of dissolved salt at some time prior to biological sampling. Other studies have also found associations of benthic macroinvertebrate community structural indices with salinity, as measured using point-in-time samples, to be statistically significant yet highly variable (e.g., Bernhardt *et al.*, 2012).

CONCLUSIONS

Through rigorous, targeted site selection, we were able to minimize the influence of salinity-covariant stressors commonly present in coal mining-influenced streams, thus effectively isolating salinity as a potential stressor to the benthic macroinvertebrate community. We found highly significant, negative, and variable relationships between an indicator of biological condition (VASCI) and salinity. Consequently, our results demonstrate that elevated salinity is an important limiting factor for biological condition in Central Appalachian headwater streams.

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