

Original Articles

Benthic macroinvertebrate community response to salinization in headwater streams in Appalachia USA over multiple years



Anthony J. Timpano^{a,*}, Stephen H. Schoenholtz^a, David J. Soucek^b, Carl E. Zipper^c

^a Virginia Water Resources Research Center, Virginia Tech, 310 West Campus Dr, RM 210, Blacksburg, VA 24061, USA

^b Illinois Natural History Survey, 1816 S. Oak St Champaign, IL 61820, USA

^c Crop and Soil Environmental Sciences, Virginia Tech, 185 Ag Quad Ln, RM 416, Blacksburg, VA 24061, USA

ARTICLE INFO

Keywords:

Freshwater salinization
Benthic macroinvertebrates
Coal mining
Conductivity
Seasonality

ABSTRACT

Salinization of freshwaters threatens aquatic ecosystems globally via effects that include reductions in benthic macroinvertebrate diversity. Enhanced understanding of salinity effects in freshwater ecosystems could aid mitigation efforts. Salinization effects on benthic macroinvertebrate community structure were quantified in a 4.5-year study of 25 headwater streams spanning a gradient of salinity in the central Appalachian mountains of USA. Community structure as sampled was strongly seasonal, justifying treatment of Spring and Fall data separately. Stream communities became increasingly different from reference condition as salinity increased, with stronger relationships between biota and salinity in Spring than in Fall. Intra-seasonal variation in community structure was also revealed across sampling dates. Genera of the order Ephemeroptera appeared as most sensitive to salinity, as indicated by rapid declines in richness and relative abundance as salinity increased. Plecoptera and Trichoptera richness and relative abundance metrics appeared as less sensitive to salinity, and some Plecoptera genera exhibited increased relative abundance at elevated salinity. Other community metrics were weakly associated with salinity and exhibited greater variability than Ephemeroptera metrics, suggesting that Ephemeroptera richness and relative abundance are sensitive indicators with which to gauge onset of salinity effects. Declines in richness and relative abundance of non-Baetidae Ephemeroptera were associated strongly with increasing salinity, with effects observed at specific conductance levels as low as 200 $\mu\text{S}/\text{cm}$ in Spring based on seasonal discrete conductivity sampling. Chronic salinization has persistent effects on community structure over multiple years, but those effects are not uniform among taxa or consistent across seasons. Our findings suggest that effective detection of the onset of community change in salinized streams is best accomplished using Spring data and focusing on community metrics that incorporate salt-sensitive Ephemeroptera taxa.

1. Introduction

Salinization of freshwaters threatens aquatic ecosystems globally, with increased dissolved concentrations of major ions (i.e., Ca^{2+} , Mg^{2+} , Na^+ , K^+ , Cl^- , SO_4^{2-} , HCO_3^-) from a variety of sources associated with alterations of benthic macroinvertebrate community structure (Cañedo-Argüelles et al., 2013). There is increasing scientific recognition of the need for water management policies to mitigate salinization impacts to freshwater biota (Cañedo-Argüelles et al., 2016). However, much remains unknown about how salinity affects aquatic communities.

In the USA, central Appalachian headwater streams are subject to long-term salinization from surface coal mining as natural waters can leach major ions from mine spoils, raising in-stream salinity in excess of

background levels for decades, at minimum (Pond et al., 2014; Evans et al., 2014). Assessment of mining-induced salinization effects has been largely conducted using field-based observational surveys, which have documented substantial alterations of the benthic macroinvertebrate community as specific conductance (SC – a salinity surrogate) increases (e.g., Paybins et al., 2000; Hartman et al., 2005; Pond et al., 2008; Gerritsen et al., 2010; Bernhardt et al., 2012). Investigations of such alterations have used differing methodologies for measuring and relating salinity stressors and biological effects, limiting comparability among studies. Biotic effect levels of SC ranging from < 200 to > 900 $\mu\text{S}/\text{cm}$ have been reported in other studies, but Timpano et al. (2015) noted that those studies varied in their choice of salinity measure (predictor), biological endpoint (response), and modeling framework.

* Corresponding author.

E-mail address: atimpano@vt.edu (A.J. Timpano).

Freshwater salinization has been associated with aquatic life impacts globally, including in South Africa (Goetsch and Palmer, 1997), Australia (Kefford, 1998; Wright et al., 2017), France (Piscart et al., 2005), Spain (García-Criado et al., 1999; Cañedo-Argüelles et al., 2012), and Germany (Arle and Wagner, 2013). In those studies, impacts were characterized as declines in one or more measures of benthic macroinvertebrate community structure, such as richness, diversity, abundance, or evenness. In addition to loss of salt-sensitive taxa, invasion of salt-tolerant non-native species was observed (Piscart et al., 2005).

Laboratory toxicity testing has shown that dissolved salts, when elevated above natural background concentrations, can cause lethal and sublethal effects to a variety of freshwater invertebrates (Chapman et al., 2000; Kennedy et al., 2003; Soucek and Kennedy, 2005). Several recent laboratory experiments have focused on measuring salt toxicity to mayflies (Insecta: Ephemeroptera), a group particularly sensitive to salinity in mining-influenced streams (Johnson et al., 2015). Chronic experiments with the mayfly *Neocloeon triangulifer* (Ephemeroptera: Baetidae) have demonstrated lethal and sublethal effects from individual salts (e.g., Johnson et al., 2015; Soucek and Dickinson, 2015), as well as from ion mixtures typical of surface waters receiving alkaline mine drainage (e.g., Kunz et al., 2013). Ongoing experimental research into ecotoxicology of salts and salt mixtures is illuminating mechanisms and responses of indicator species, but field-based data form the basis of current efforts to establish salinity “benchmarks”- minimum SC levels at which biotic community alterations from a reference condition are observed (Cormier et al., 2013). Therefore, quantifying field-based salinity sensitivity is an important complement to laboratory experimentation as a means for gaining improved understanding of salinity effects on benthic macroinvertebrates.

Biological assessments commonly use benthic macroinvertebrates as indicators; results of such assessments can vary based on taxonomic composition of samples, response metrics derived therefrom, and choice of metric endpoint used to define effects (Cao and Hawkins, 2011). In addition, choice of SC predictor and modeling framework can influence conclusions drawn regarding salinity levels associated with specific biological effects. Therefore, we sought to assess salinity-biota relationships while accounting for sources of variation in water quality of headwater streams subjected to mining influence. Our objectives were to characterize response to salinity of benthic macroinvertebrates at the community level, identify relative salt-sensitivity of individual genera and taxonomic groups, model salinity response of the most salt-sensitive community metrics, and determine minimum SC levels associated with community change.

2. Methods

2.1. Stream selection

Twenty-five forested first-order streams were selected for assessment of SC and benthic macroinvertebrates in the central Appalachian coalfield of Virginia and West Virginia. Streams were selected to minimize influence by non-salinity stressors on benthic macroinvertebrate communities (Timpano et al., 2015). Twenty streams with mining-induced salinity elevated above background were considered “test” streams, and five streams with minimal disturbance and no mining were considered “reference” streams. See Timpano et al. (2015, 2018) for details regarding stream selection criteria, selection methods, and stream attributes.

2.2. Benthic macroinvertebrates

We surveyed the benthic macroinvertebrate community in each stream over a 4.5-year period during Fall (2011, 2012, 2013, 2015) and Spring (2012, 2013, 2014, 2016) seasons. Timing of sample collection was consistent during each season across years, with most samples

collected during April (median date = 16 April) and October (median date = 20 October). Samples from all streams were generally collected within approximately nine days of one another during a season. Exact timing of biological sampling within a season was influenced strongly by stream flow, with no sampling occurring during flow extremes. We took care to collect all samples during baseflow (i.e., stream flow not influenced by storm flow) and at least seven days after extreme flows that were estimated to have caused scouring. Baseflow sampling helped to ensure adequate time for recolonization of substrate after high flow and allowed for concurrent collection of baseflow water samples and measurement of SC.

Samples were collected using the single-habitat method for high-gradient streams as per U.S. EPA Rapid Bioassessment Protocols (Barbour et al., 1999). Using a 0.3-m D-frame kicknet with 500- μ m mesh, a single composite sample (approximately 2 m²) composed of six 1 \times 0.3-m kicks was collected along a 100-m reach at each stream. Because of presence of Endangered Species Act-listed crustaceans and mollusks in the region, all specimens from those groups were returned to the streams unharmed. Samples were preserved in 95% ethanol and returned to the laboratory for sorting and identification.

Biological samples were sub-sampled randomly to obtain a 200 (\pm 10%) organism count following Virginia Department of Environmental Quality methods (VDEQ, 2008), which are adapted from RBP methods (Barbour et al., 1999) and are comparable to methods used by West Virginia Department of Environmental Protection (WVDEP, 2015). Specimens were identified to genus/lowest practicable level using standard keys (Stewart et al., 1993; Wiggins, 1996; Smith, 2001; Merritt et al., 2008), except individuals in family Chironomidae and subclass Oligochaeta, which were identified at those levels.

2.3. Stream water chemistry

Water temperature, dissolved oxygen, SC, and pH were measured *in situ* at baseflow in Spring and Fall at the time of biological sample collection with a calibrated handheld multi-probe meter (Hanna HI-9828 - Hanna Instruments, Inc., Woonsocket, Rhode Island, USA; or YSI Professional Plus - YSI, Inc., Yellow Springs, Ohio, USA). To assess dissolved ion concentrations, grab-samples of water were collected approximately monthly (\leq 19 times) at each stream during baseflow (i.e., stream flow not influenced by storm flow) from May 2011 through April 2013, as well as concurrently with biological sampling. Stream water samples were filtered immediately using polyvinylidene difluoride syringe filters with a nominal pore size of 0.45 μ m and stored in sterile polyethylene sample bags. Aliquots for analysis of cations were preserved to pH < 2 with 1 + 1 concentrated ultrapure nitric acid. All samples were transported to the laboratory on ice and stored at 4 °C until analysis.

In the laboratory, water samples were analyzed for major cations (Ca²⁺, Mg²⁺, K⁺, Na⁺), and dissolved trace elements (Al, Cu, Fe, Mn, Se, Zn) using either an inductively coupled plasma-optical emission spectrometer (Varian Vista MPX ICP-OES w/ICP Expert software, Varian Instruments, Walnut Creek, California USA) or an inductively coupled plasma-mass spectrometer (Thermo Electron X-Series ICP-MS, Thermo Fisher Scientific, Waltham, Massachusetts USA) (APHA, 2005). An ion chromatograph (Dionex DX500, Dionex Corp., Sunnyvale, California USA) was used to measure Cl⁻ and SO₄²⁻ (APHA, 2005). Total alkalinity was measured by titration with standard acid (APHA, 2005) using a potentiometric auto-titrator (TitraLab 865, Radiometer Analytical, Lyon, France). Concentrations of the anions CO₃²⁻ and HCO₃⁻ were calculated from alkalinity and pH measurements (APHA, 2005). Water chemistry data were examined to determine if trace elements were present at chronically toxic levels (exceeding criteria continuous concentrations [CCC]) (USEPA, 2012, 2016; ILEPA, 2001). This was done to determine if there was potential for trace element toxicity to confound observed salinity-biota associations.

2.4. Data analysis

2.4.1. Conductivity data

We evaluated salinity-benthic macroinvertebrate relationships using SC measured *in situ* at the time of benthic macroinvertebrate sample collection, with data from each sample season and year (e.g., Spring 2012) treated discretely (i.e., no pooling or averaging of data across years/seasons). Our sampling approach is consistent with the majority of salinity-benthic macroinvertebrate investigations conducted in the Appalachian coalfield region during the past two decades, which use discrete data collected infrequently, often just one time (e.g., Bernhardt et al., 2012) or once or twice per year (e.g., Green et al., 2000; Timpano et al., 2015). Discrete data also form the basis for recent efforts to develop field-based salinity standards for freshwater aquatic life protection (Cormier et al., 2013).

2.4.2. Community- and taxon-level responses to salinity

We evaluated response of the benthic macroinvertebrate community to salinity by indirect gradient analysis using non-metric multidimensional scaling (NMDS) (McCune et al., 2002; Legendre and Legendre, 2012). We rotated the ordination to align NMDS Axis 1 with SC and calculated correlation coefficients between SC and sample locations in ordination space to provide a magnitude and direction of association between those factors and community differences (Legendre and Legendre, 2012).

Prior to ordination, raw taxa abundance values were transformed to proportional abundance and used to calculate Bray-Curtis dissimilarities among streams. Three ordinations (dimension = 2) were computed using (1) data from both seasons (BOTH model; eight samples from six calendar years, $n = 194$ samples), (2) only Spring data (SPRING model; four years, $n = 99$ samples), and (3) only Fall data (FALL model; four years, $n = 95$ samples).

We omitted from multivariate analyses taxa that were found in fewer than 5% of samples (i.e., “rare” taxa), as determined separately for each of the three models. Rare taxa for the BOTH model were those found in fewer than 10 samples and rare taxa for the SPRING and FALL models were those found in fewer than five samples. We chose to exclude rare taxa to reduce likelihood that observed taxon absence was a result of sampling bias (Cormier et al., 2013), transient presence at a sampling reach, or other chance occurrence (Legendre and Legendre, 2012).

Seasonal differences in the composition of benthic macroinvertebrate samples can occur as a result of natural seasonal variation in life histories among taxa common in headwater streams of our study region (Poff et al., 2006; Merritt et al., 2008; Pond and North, 2013). Such differences in sample composition reflect seasonal changes in abundance of mature specimens and associated probabilities of capture for each taxon. Therefore, we refer to community structure and its seasonal differences as representing these natural seasonal changes in community-sample composition, rather than wholesale shifts in stream community taxa between seasons.

We describe biological responses to salinity as sensitive or tolerant in an operational context. Salinity may have a direct physiological effect on a taxon, such as mortality, but salinity could also cause indirect ecological effects to a given taxon. Indirect ecological mechanisms may include changes in predation or competition that can result from salinity acting directly on other taxa (Kefford et al., 2004b). Therefore, we use the terms sensitive and tolerant operationally, to include both potential direct physiological effects, as well as potential indirect ecological effects.

To account for natural seasonal variation of macroinvertebrate sample composition, we first used the BOTH model to determine if community structure differed by season. Next, we used the SPRING and FALL models separately to quantify association between salinity and change at the community level by rotating ordinations to align NMDS Axis 1 with the conductivity gradient and computing Pearson

correlation coefficients of stream scores with SC along NMDS Axis 1 (Legendre and Legendre, 2012). We then ranked taxon scores along NMDS Axis 1 and summarized ranks at the genus, family, and order levels to determine relative sensitivity to salinity of individual taxa and taxonomic groups. Lower taxon scores along NMDS Axis 1 are associated with low-SC reference streams and higher taxon scores along that axis are associated with higher-SC test streams. Taxon score rank serves as a proxy for SC sensitivity because NMDS Axis 1 is parallel to and correlated with the SC gradient. Relative sensitivities of individual taxa were determined directly based on taxon score rank (i.e., the taxon with rank 1 is most sensitive to SC). Family and order sensitivities were determined by calculating the median rank of individual taxa belonging to each taxonomic group of interest, with lower median rank values indicating higher sensitivity to SC.

2.4.3. Community metrics

Benthic macroinvertebrate community structure was quantified by calculating 25 candidate metrics that are often used for biological assessment (Barbour et al., 1999) and that summarize elements of community richness, composition, diversity, evenness, and trophic functional traits (Table S1). Candidate taxa were screened for responsiveness to salinity and a subset of the 25 metrics were used for further modeling, as described below.

We characterized response of community metrics to SC by fitting generalized additive mixed models (GAMM) relating each community metric to SC, with separate models for Spring and Fall data. We modeled SC as a fixed effect and stream as a random effect to account for multiple samples from each stream during the study period. We fit models using the Poisson error distribution with log-link for richness metrics (i.e., count data) and the Gaussian distribution with identity-link (i.e., continuous data) for all others (Quinn and Keough, 2002). All community metrics with significant GAMMs ($p < 0.05$) and R^2 values ≥ 0.5 were retained for final modeling. Several additional metrics with significant relationships with SC ($p < 0.05$) and GAMM R^2 of approximately 0.40 were included, as they represented common measures of community richness, diversity, and evenness, bringing the total number of community metrics evaluated to nine in Spring and six in Fall.

2.4.4. Community change and critical conductivity levels

To identify minimum levels of salinity (critical conductivity, SC_{crit}) associated with change in community structure, we first defined change as deviation from reference condition. This is a common approach to defining reference thresholds, which we inferred to be the value of each metric at a high or low quantile (e.g., 10th percentile) of its reference-stream distribution (Hawkins et al., 2010). For metrics expected to decline with increasing perturbation, the reference threshold was the 10th percentile of reference-stream metric values, and for metrics that increased with salinity, the reference threshold was the 90th percentile of reference values. Reference thresholds were determined for each season using pooled data from the full study period. Critical conductivity levels were defined for each community metric as the SC associated with the intersection of the reference threshold with a fitted GAMM curve. Upper and lower 95% confidence limits of SC_{crit} were defined as the SC at which GAMM 95% confidence limit curves crossed the reference threshold. We fit GAMMs and calculated SC_{crit} using nine community metrics that are responsive to salinity in Spring, and six metrics that are responsive to salinity in Fall (Table S1).

All analyses were conducted using R statistical software version 3.4.0 (R Core Team, 2017). Multivariate ordination used the package *vegan* (Oksanen et al., 2017) and generalized additive mixed modeling used the package *gamm4* (Wood and Scheipl, 2016).

3. Results

3.1. Stream water chemistry

The dominant dissolved cation was Ca^{2+} in both stream types (mean molar proportion = 16.5% in reference streams, 19.7% in test streams), with Mg^{2+} proportion comparable to Ca^{2+} in test streams. The dominant anion in test streams was SO_4^{2-} (27.6%), whereas HCO_3^- was the primary anion in reference streams (34.9%). No sites were dominated by Na^+ or Cl^- . Overall ion proportions were generally comparable along the salinity gradient with the exception that ratios of $\text{SO}_4:\text{HCO}_3$ and $\text{Mg}:\text{Ca}$ increased with SC, as is typical for streams of the region influenced by surface mining (Timpano et al., 2017). See Timpano et al. (2018) for more details regarding ion proportions.

Evaluation of dissolved trace elements ($n = 92$ samples) did not suggest that salinity-biota models were confounded by toxic effects from trace metals. Aluminum, Cu, and Fe were less than U.S. EPA aquatic-life CCCs (USEPA, 2012) in all samples and Zn was greater than CCC in only one sample (data not shown). There is no U.S. EPA aquatic-life water-quality standard for Mn, but all values reported here were at least one order of magnitude lower than the proposed Illinois EPA aquatic-life CCC (ILEPA, 2001), which is the only candidate aquatic-life criterion for Mn that we were able to identify. Selenium concentration was less than detection limit ($< 2.5 \mu\text{g Se/L}$) in 76 samples but was consistently greater than the U.S. EPA aquatic-life CCC of $3.1 \mu\text{g Se/L}$ (USEPA, 2016) in three streams (12 samples) and measured $> 3.1 \mu\text{g Se/L}$ once in each of four other streams. Selenium was not significantly correlated with SC in samples where it was detected at quantifiable levels ($n = 16$; Spearman's $\rho = -0.0707$, $p = 0.7947$, data not shown).

3.2. Occurrence of benthic macroinvertebrate taxa

A total of 115 taxa were observed in 194 samples collected from Fall 2011 through Spring 2016 (Table S2). Fall had higher richness of benthic macroinvertebrates, with 101 taxa ($n = 95$ samples), as compared to only 89 taxa found in Spring ($n = 99$ samples). The five most-common taxa across all seasons were Chironomidae (192 samples), *Diplectrona* (189), *Leuctra* (161), *Rhyacophila* (157), and *Ectopria* (135). Genera *Diplectrona* and *Leuctra* also represented the most-common genus in their order (Trichoptera and Plecoptera, respectively), whereas *Baetis* was the most-common Ephemeropteran genus (114 samples). *Ephemerella* was the next most-common mayfly, found in 68 samples. There were 15 taxa found only in Spring samples and 26 taxa unique to Fall samples. The most-common taxon found only in Spring was *Drunella* (51 samples) and the most-common taxon found only in Fall was *Paracaptia* (35 samples).

Benthic macroinvertebrate taxa in reference streams were less unique compared to test streams, with only six taxa exclusive to reference streams, whereas test streams had 29 unique taxa. Among taxa segregated by stream type, the most-common taxon found only at reference streams was *Agapetus* (7 of 37 samples) and the most-common taxon unique to test streams was *Neotrichia* (16 of 157 samples). Approximately 35–45% of taxa were considered rare (found in $< 5\%$ of samples) for the purpose of NMDS ordination, resulting in 63 taxa retained for the BOTH NMDS model, with 58 and 61 taxa retained for the SPRING and FALL NMDS models, respectively (Table S2).

3.3. Community changes and salt-sensitive taxa

Distinct seasonal differences among benthic macroinvertebrate community samples were revealed by the BOTH model ordination (Fig. 1). After rotation to align NMDS Axis 1 with seasonal separation, stream ordination scores were strongly correlated with Julian day of sample collection (Fig. 1). Absence of overlap in 95% confidence ellipses for each season justified conducting an assessment of salinity-biota associations using separate seasonal models. Ordination of Spring

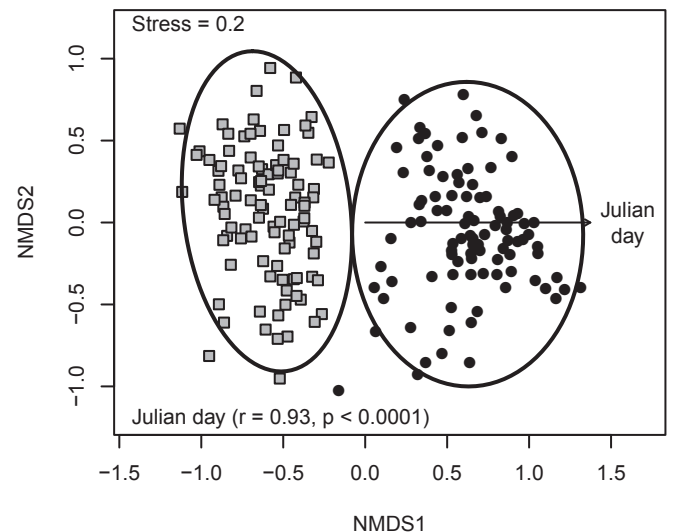


Fig. 1. NMDS plot of BOTH seasons ($n = 194$). Points are site scores for Spring (gray squares) and Fall (black circles) samples based on Bray-Curtis dissimilarity of proportional abundance of genera found in $\geq 95\%$ of samples. Lines are 95% confidence ellipses around seasons. Arrow indicates direction of Pearson correlation between NMDS Axis 1 and Julian day of sample collection.

and Fall seasons separately indicated that benthic macroinvertebrate community structure of salinized streams was increasingly different from reference stream community structure as SC increased. Correlations of NMDS Axis 1 with $\log_{10}(\text{SC})$ were nominally higher in Spring (Fig. 2A) than in Fall (Fig. 2B) models. Community structure also differed among streams within sample seasons based on when samples were collected. Correlations between Julian day of sample collection and ordination location were moderate for Spring samples (Fig. 2A) but weaker for Fall samples (Fig. 2B).

3.4. Identifying salt-sensitive taxa

Mayflies (order Ephemeroptera) were the group most sensitive (i.e., associated with lower-salinity streams) to SC in both seasons, as indicated by highest median rank of NMDS Axis 1 scores for Ephemeroptera genera (Tables 1 and 2). Mayflies accounted for three and four of the 10 most-sensitive families in Spring (Table 1) and Fall (Table 2), respectively. Notably less sensitive than other mayflies was the family Baetidae, which had median rank of 29 in Spring (Table 1) and 39 in Fall (Table 2). Further, mayfly genera constitute eight and nine of the 20 most-sensitive taxa in Spring (Table 3) and Fall (Table 4), respectively, with the seven most-sensitive taxa in Fall belonging to that order (Table 4). In both seasons, stoneflies (order Plecoptera) and caddisflies (order Trichoptera) were less sensitive than mayflies but more sensitive than true flies (order Diptera), the next most-common order (Tables 1 and 2).

Among the groups considered sensitive to perturbation, several stonefly and caddisfly genera appeared to be more tolerant of salinity than their order-rank would suggest. Of the stoneflies, genera *Amphinemura* and *Leuctra* had low salt sensitivity in Spring samples, ranking 53 and 56, respectively (Table 3). In Fall, *Allocapnia* also tolerated high salinity, with a rank of 60 (Table 4). All three taxa were ubiquitous, being found in nearly all streams. Caddisflies of the family Hydropsychidae (genera *Hydropsyche*, *Ceratopsyche*, *Diplectrona*, and *Cheumatopsyche*) were all relatively salt-tolerant (i.e., associated with higher-salinity streams), ranking below the order-rank in both Spring (Table 3) and Fall (Table 4).

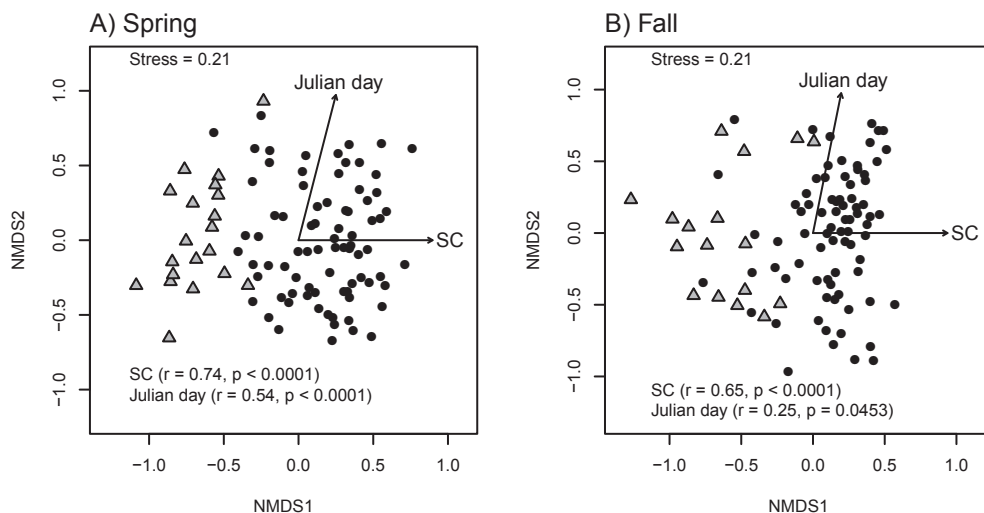


Fig. 2. NMDS plot of A) SPRING and B) FALL samples. Arrows indicate direction of Pearson correlations of samples with $\log_{10}(\text{SC})$ and Julian day of sample collection. Points are site scores for reference streams (triangles) and test streams (circles) based on Bray-Curtis dissimilarity of proportional abundance of genera found in $\geq 95\%$ of samples for each season.

3.5. Community metrics and critical conductivity levels

3.5.1. Salinity-biota relationships

Models of community metrics as a function of SC were significant (GAMM $p < 0.05$) for 19 metrics in Spring and 17 metrics in Fall (Table S1). Of those models, nine met selection criteria in Spring (Fig. 3), and six did so in Fall (Fig. 4). Those metrics were retained for modeling with SC and determination of critical conductivity levels.

Metrics heavily influenced by Ephemeroptera taxa were most strongly responsive to SC, exhibiting steady decline with increasing SC, then decreasing more slowly above approximately 500 $\mu\text{S}/\text{cm}$ in Spring (Fig. 3A–D, F) and above approximately 1000 $\mu\text{S}/\text{cm}$ in Fall (Fig. 4A–E). Shannon diversity, as well as the evenness measure % 5 Dominant Taxa,

declined with increased SC in a non-linear manner in both seasons, but relationships were weaker for those metrics, as model R^2 were < 0.50 in Spring (Fig. 3H–I) and < 0.40 in Fall (data not shown). Richness of insect orders other than Ephemeroptera in both seasons was either weakly responsive to SC (Plecoptera Richness $R^2 \leq 0.28$; data not shown), or models were not significant (Coleoptera Richness, Diptera Richness, and Trichoptera Richness $p > 0.05$; data not shown).

3.5.2. Critical conductivity levels

Critical conductivity (SC_{crit}) varied among community metrics, ranging from approximately 200–400 $\mu\text{S}/\text{cm}$ in Spring (Fig. 3) and from approximately 240–600 $\mu\text{S}/\text{cm}$ in Fall (Fig. 4). Models with higher GAMM R^2 generally had lower and more precise (i.e., narrower 95%

Table 1
 Rank of relative salinity sensitivity by family and order for Spring samples.

| Order | Family | Median Genus Rank | No. Genera | Order | Median Genus Rank | No. Genera |
|---------------|-------------------|-------------------|------------|---------------|-------------------|------------|
| Ephemeroptera | Leptophlebiidae | 4 | 1 | Ephemeroptera | 14 | 11 |
| Trichoptera | Glossosomatidae | 8 | 2 | Coleoptera | 26.5 | 4 |
| Plecoptera | Pteronarcyidae | 9 | 1 | Plecoptera | 32.5 | 12 |
| Ephemeroptera | Heptageniidae | 9.5 | 4 | Trichoptera | 35 | 13 |
| Trichoptera | Uenoidae | 11 | 1 | Diptera | 39 | 15 |
| Ephemeroptera | Ephemerellidae | 13 | 2 | Odonata | 49 | 1 |
| Diptera | Ceratopogonidae | 17 | 3 | Oligochaeta | 51 | 1* |
| Plecoptera | Perlodidae | 20 | 3 | Megaloptera | 52 | 1 |
| Trichoptera | Lepidostomatidae | 21 | 1 | | | |
| Coleoptera | Psephenidae | 22.5 | 2 | | | |
| Trichoptera | Philopotamidae | 26.5 | 2 | | | |
| Diptera | Tipulidae | 27 | 4 | | | |
| Coleoptera | Elmidae | 27.5 | 2 | | | |
| Diptera | Simuliidae | 28.5 | 2 | | | |
| Ephemeroptera | Baetidae | 29 | 3 | | | |
| Plecoptera | Chloroperlidae | 29 | 2 | | | |
| Ephemeroptera | Ameletidae | 30 | 1 | | | |
| Plecoptera | Peltoperlidae | 34 | 2 | | | |
| Trichoptera | Polycentropodidae | 35 | 1 | | | |
| Diptera | Chironomidae | 40 | 1* | | | |
| Diptera | Empididae | 41 | 4 | | | |
| Plecoptera | Perlidae | 41 | 2 | | | |
| Trichoptera | Rhyacophilidae | 41 | 1 | | | |
| Trichoptera | Hydropsychidae | 44.5 | 4 | | | |
| Diptera | Dixidae | 46 | 1 | | | |
| Odonata | Gomphidae | 49 | 1 | | | |
| | Oligochaeta | 51 | 1* | | | |
| Megaloptera | Corydalidae | 52 | 1 | | | |
| Plecoptera | Nemouridae | 53 | 1 | | | |
| Plecoptera | Leuctridae | 56 | 1 | | | |
| Trichoptera | Hydroptilidae | 57 | 1 | | | |

* Specimens identified to family or subclass as named.

Table 2
Rank of relative salinity sensitivity by family and order for Fall samples.

| Order | Family | Median Genus Rank | No. Genera | Order | Median Genus Rank | No. Genera |
|---------------|-------------------|-------------------|------------|---------------|-------------------|------------|
| Ephemeroptera | Ephemereilidae | 2 | 3 | Ephemeroptera | 6.5 | 12 |
| Ephemeroptera | Leptophlebiidae | 3 | 1 | Trichoptera | 29.5 | 14 |
| Ephemeroptera | Ephemeridae | 4 | 1 | Plecoptera | 30 | 13 |
| Plecoptera | Limnephilidae | 9 | 1 | Diptera | 31 | 14 |
| Ephemeroptera | Heptageniidae | 9.5 | 4 | Oligochaeta | 41 | 1 |
| Plecoptera | Perlodidae | 15.5 | 2 | Odonata | 49 | 1 |
| Plecoptera | Pteronarcyidae | 17 | 1 | Megaloptera | 50 | 2 |
| Trichoptera | Uenoidae | 20 | 1 | Coleoptera | 53.5 | 4 |
| Diptera | Chironomidae | 21 | 1* | | | |
| Diptera | Ceratopogonidae | 22 | 3 | | | |
| Plecoptera | Perlidae | 23.5 | 2 | | | |
| Trichoptera | Psychomyiidae | 25 | 1 | | | |
| Diptera | Tipulidae | 26 | 5 | | | |
| Trichoptera | Philopotamidae | 27 | 3 | | | |
| Trichoptera | Polycentropodidae | 27.5 | 2 | | | |
| Plecoptera | Chloroperlidae | 31 | 2 | | | |
| Ephemeroptera | Ameletidae | 34 | 1 | | | |
| Plecoptera | Capniidae | 35 | 2 | | | |
| Diptera | Dixidae | 36 | 1 | | | |
| Ephemeroptera | Baetidae | 39 | 2 | | | |
| Diptera | Empididae | 39 | 3 | | | |
| Diptera | Simuliidae | 40 | 1 | | | |
| | Oligochaeta | 41 | 1* | | | |
| Plecoptera | Peltoperlidae | 42 | 1 | | | |
| Megaloptera | Corydalidae | 43 | 1 | | | |
| Trichoptera | Hydropsychidae | 45 | 4 | | | |
| Plecoptera | Taeniopterygidae | 46 | 1 | | | |
| Trichoptera | Rhyacophilidae | 47 | 1 | | | |
| Plecoptera | Leuctridae | 48 | 1 | | | |
| Coleoptera | Psephenidae | 48 | 2 | | | |
| Odonata | Gomphidae | 49 | 1 | | | |
| Trichoptera | Hydroptilidae | 53 | 1 | | | |
| Coleoptera | Elmidae | 53.5 | 2 | | | |
| Megaloptera | Sialidae | 57 | 1 | | | |
| Plecoptera | Nemouridae | 61 | 1 | | | |

* Specimens identified to family or subclass as named.

Table 3
Taxon relative salinity sensitivity by decreasing rank for Spring samples.

| Order ¹ | Taxon ² | NMDS Axis 1 Score | No. Samples | Rank | Order ¹ | Taxon ² | NMDS Axis 1 Score | No. Samples | Rank |
|--------------------|-------------------------|-------------------|-------------|------|--------------------|-----------------------|-------------------|-------------|------|
| T | <i>Agapetus</i> | -1.3373 | 7 | 1 | E | <i>Ameletus</i> | -0.2368 | 27 | 30 |
| D | <i>Prosimulium</i> | -1.3203 | 14 | 2 | T | <i>Wormaldia</i> | -0.2312 | 27 | 31 |
| P | <i>Remenus</i> | -1.2137 | 7 | 3 | P | <i>Peltoperla</i> | -0.2298 | 20 | 32 |
| E | <i>Paraleptophlebia</i> | -1.1105 | 20 | 4 | P | <i>Haploperla</i> | -0.2284 | 37 | 33 |
| E | <i>Cinygmula</i> | -1.0916 | 20 | 5 | P | <i>Isoperla</i> | -0.2283 | 50 | 34 |
| E | <i>Epeorus</i> | -1.0644 | 42 | 6 | T | <i>Polycentropus</i> | -0.1972 | 21 | 35 |
| D | <i>Hexatoma</i> | -0.9433 | 26 | 7 | P | <i>Tallaperla</i> | -0.1930 | 24 | 36 |
| E | <i>Ephemerella</i> | -0.9133 | 57 | 8 | D | <i>Chelifera</i> | -0.1439 | 61 | 37 |
| P | <i>Pteronarcys</i> | -0.8677 | 24 | 9 | T | <i>Hydropsyche</i> | -0.1280 | 24 | 38 |
| D | <i>Dasyhelea</i> | -0.8188 | 6 | 10 | D | <i>Hemerodromia</i> | -0.1225 | 35 | 39 |
| T | <i>Neophylax</i> | -0.7092 | 19 | 11 | D | Chironomidae | -0.1101 | 97 | 40 |
| D | <i>Dicranota</i> | -0.6889 | 8 | 12 | T | <i>Rhyacophila</i> | -0.0853 | 73 | 41 |
| E | <i>Stenonema</i> | -0.6527 | 9 | 13 | D | <i>Antocha</i> | -0.0529 | 9 | 42 |
| E | <i>Dipheter</i> | -0.6363 | 11 | 14 | D | <i>Neoplaista</i> | -0.0502 | 15 | 43 |
| T | <i>Glossosoma</i> | -0.5501 | 5 | 15 | T | <i>Ceratopsyche</i> | -0.0420 | 37 | 44 |
| E | <i>Maccaffertium</i> | -0.5161 | 14 | 16 | T | <i>Diplectrona</i> | -0.0364 | 96 | 45 |
| D | <i>Bezzia</i> | -0.4484 | 30 | 17 | D | <i>Dixa</i> | 0.0120 | 11 | 46 |
| E | <i>Drunella</i> | -0.4473 | 51 | 18 | D | <i>Clinocera</i> | 0.0525 | 18 | 47 |
| C | <i>Ectopria</i> | -0.4417 | 69 | 19 | E | <i>Baetis</i> | 0.0710 | 73 | 48 |
| P | <i>Yugus</i> | -0.4238 | 29 | 20 | O | <i>Lanthus</i> | 0.0827 | 41 | 49 |
| T | <i>Lepidostoma</i> | -0.3862 | 10 | 21 | D | <i>Tipula</i> | 0.1294 | 20 | 50 |
| T | <i>Dolophilodes</i> | -0.3673 | 33 | 22 | | Oligochaeta | 0.1332 | 49 | 51 |
| D | <i>Stilobezzia</i> | -0.3552 | 5 | 23 | M | <i>Nigronia</i> | 0.2059 | 15 | 52 |
| P | <i>Acroneuria</i> | -0.3535 | 41 | 24 | P | <i>Amphinemura</i> | 0.3088 | 99 | 53 |
| P | <i>Sweltsa</i> | -0.3481 | 19 | 25 | T | <i>Cheumatopsyche</i> | 0.3399 | 44 | 54 |
| C | <i>Psephenus</i> | -0.3424 | 18 | 26 | D | <i>Simulium</i> | 0.4228 | 54 | 55 |
| C | <i>Oulimnius</i> | -0.3157 | 54 | 27 | P | <i>Leuctra</i> | 0.4941 | 98 | 56 |
| C | <i>Optioservus</i> | -0.3048 | 39 | 28 | T | <i>Ochrotrichia</i> | 0.5427 | 5 | 57 |
| E | <i>Acentrella</i> | -0.2807 | 49 | 29 | P | <i>Perlesta</i> | 0.6062 | 7 | 58 |

1 C: Coleoptera, D: Diptera, E: Ephemeroptera, M: Megaloptera, O: Odonata, P: Plecoptera, T: Trichoptera.

2 Taxa identified to genus, except Chironomidae and Oligochaeta; those specimens at family or subclass as named.

Table 4
Taxon relative salinity sensitivity by decreasing rank for Fall samples.

| Order ¹ | Taxon ² | NMDS Axis 1 Score | No. Samples | Rank | Order ¹ | Taxon ² | NMDS Axis 1 Score | No. Samples | Rank |
|--------------------|-------------------------|-------------------|-------------|------|--------------------|-----------------------|-------------------|-------------|------|
| E | <i>Eurylophella</i> | -1.8550 | 13 | 1 | P | <i>Sweltsa</i> | -0.1603 | 27 | 32 |
| E | <i>Attenella</i> | -1.5571 | 9 | 2 | E | <i>Dipheter</i> | -0.1588 | 9 | 33 |
| E | <i>Paraleptophlebia</i> | -1.4600 | 19 | 3 | E | <i>Ameletus</i> | -0.1548 | 13 | 34 |
| E | <i>Ephemera</i> | -1.1422 | 9 | 4 | T | <i>Ceratopsyche</i> | -0.1348 | 50 | 35 |
| E | <i>Ephemerella</i> | -1.0601 | 11 | 5 | D | <i>Dixa</i> | -0.1175 | 26 | 36 |
| E | <i>Epeorus</i> | -1.0587 | 13 | 6 | C | <i>Ectopria</i> | -0.0895 | 66 | 37 |
| E | <i>Stenacron</i> | -1.0433 | 7 | 7 | T | <i>Cheumatopsyche</i> | -0.0766 | 67 | 38 |
| P | <i>Yugus</i> | -0.9179 | 8 | 8 | D | <i>Clinocera</i> | -0.0500 | 5 | 39 |
| T | <i>Pycnopsyche</i> | -0.9041 | 24 | 9 | D | <i>Simulium</i> | -0.0468 | 29 | 40 |
| P | <i>Paracapnia</i> | -0.8911 | 35 | 10 | | <i>Oligochaeta</i> | -0.0216 | 31 | 41 |
| D | <i>Dasyhelea</i> | -0.8656 | 8 | 11 | P | <i>Peltoperla</i> | -0.0210 | 27 | 42 |
| E | <i>Stenonema</i> | -0.8226 | 12 | 12 | M | <i>Nigronia</i> | -0.0104 | 19 | 43 |
| D | <i>Chimarra</i> | -0.7327 | 16 | 13 | D | <i>Tipula</i> | -0.0033 | 70 | 44 |
| D | <i>Chelifera</i> | -0.7280 | 7 | 14 | E | <i>Baetis</i> | 0.0005 | 41 | 45 |
| D | <i>Hexatoma</i> | -0.6115 | 32 | 15 | P | <i>Taeniopteryx</i> | 0.0062 | 31 | 46 |
| E | <i>Maccaffertium</i> | -0.5840 | 35 | 16 | T | <i>Rhyacophila</i> | 0.0122 | 84 | 47 |
| P | <i>Pteronarcys</i> | -0.5632 | 17 | 17 | P | <i>Leuctra</i> | 0.0130 | 63 | 48 |
| P | <i>Eccopectura</i> | -0.4269 | 6 | 18 | O | <i>Lanthus</i> | 0.0295 | 48 | 49 |
| D | <i>Limnophila</i> | -0.4253 | 6 | 19 | D | <i>Hemerodromia</i> | 0.0458 | 23 | 50 |
| T | <i>Neophylax</i> | -0.4100 | 17 | 20 | C | <i>Oulimnius</i> | 0.0797 | 51 | 51 |
| D | Chironomidae | -0.3913 | 95 | 21 | T | <i>Hydropsyche</i> | 0.0899 | 54 | 52 |
| D | <i>Bezzia</i> | -0.3708 | 33 | 22 | T | <i>Neotrichia</i> | 0.2062 | 16 | 53 |
| P | <i>Isoperla</i> | -0.3549 | 27 | 23 | D | <i>Antocha</i> | 0.2090 | 9 | 54 |
| T | <i>Cynnellus</i> | -0.3430 | 11 | 24 | T | <i>Diplectrona</i> | 0.2339 | 93 | 55 |
| T | <i>Lype</i> | -0.3415 | 7 | 25 | C | <i>Optioservus</i> | 0.2390 | 43 | 56 |
| D | <i>Dicranota</i> | -0.2972 | 13 | 26 | M | <i>Sialis</i> | 0.3272 | 5 | 57 |
| T | <i>Wormaldia</i> | -0.2857 | 37 | 27 | D | <i>Forcipomyia</i> | 0.3309 | 11 | 58 |
| T | <i>Dolophilodes</i> | -0.2409 | 58 | 28 | C | <i>Psephenus</i> | 0.3627 | 33 | 59 |
| P | <i>Acroneuria</i> | -0.2291 | 49 | 29 | P | <i>Allocapnia</i> | 0.3716 | 91 | 60 |
| P | <i>Haploperla</i> | -0.1966 | 20 | 30 | P | <i>Soyedina</i> | 0.4084 | 5 | 61 |
| T | <i>Polycentropus</i> | -0.1701 | 31 | 31 | | | | | |

1 C: Coleoptera, D: Diptera, E: Ephemeroptera, M: Megaloptera, O: Odonata, P: Plecoptera, T: Trichoptera.

2 Taxa identified to genus, except Chironomidae and Oligochaeta; those specimens at family or subclass as named.

confidence intervals) values for SC_{crit} (Figs. 3 and 4). Metrics for sensitive mayflies (i.e., metrics excluding Baetidae) had the lowest SC_{crit} levels and highest precision in both seasons, but Spring was lowest overall, with $SC_{crit} = 214 \mu\text{S}/\text{cm}$ for sensitive mayfly richness (Fig. 3A) and $SC_{crit} = 196 \mu\text{S}/\text{cm}$ for sensitive mayfly relative abundance (Fig. 3B). Reference thresholds were also lower in Fall than in Spring, indicating that SC_{crit} values are not directly comparable between seasons as they correspond to different seasonal reference conditions.

4. Discussion

4.1. Biological response to salinity

Overall benthic macroinvertebrate community structure as measured by multivariate ordination was increasingly dissimilar from reference condition as salinity increased. Deviation from reference condition was also observed along the salinity gradient for all nine metrics of community structure used in final analyses. These general responses are consistent with those observed in other studies of salinized central Appalachian headwater streams using similar community metrics (Green et al., 2000; Pond, 2004; Pond et al., 2008; Merriam et al., 2011; Bernhardt et al., 2012) and using multivariate analyses at the genus level (Pond, 2010, 2012; Pond et al., 2014). However, our results indicate that salinity effects are unequal among genera and order-level aggregate metrics, suggesting that community changes are driven by disproportionate salinity effects to certain taxa, rather than broad community-wide effects.

Community-metric response may have also been influenced by any temporal trends in salinity during the study period. However, results from a prior analysis indicated no consistent evidence of significant trends in salinity, biology, or ion matrix composition over multiple years in our study streams (Timpano et al., 2017).

Our assessment of relative salt sensitivity of individual taxa agrees with findings by others that Ephemeroptera is the order most sensitive to salinity in Appalachian coalfield streams (Pond, 2004, 2010; Hartman et al., 2005; Pond et al., 2008, 2014), but not all Ephemeroptera responded equally to SC. Members of family Baetidae appear more tolerant of coal-mining induced salinization than other Ephemeroptera (Pond, 2010). Our data suggest the same, as we found that in Spring the Baetidae genera *Acentrella* and *Baetis* composed the bulk of Ephemeroptera specimens in test streams (median = 77.5%; data not shown), but were relatively uncommon in reference streams (median = 7.43%; data not shown). However, Baetidae abundance declined at higher SC, and coupled with decreases in other salt-sensitive Ephemeroptera taxa, the metric % Ephemeroptera exhibited a moderately strong negative salinity response. The strongest SC-response signals of all community metrics were found with Ephemeroptera metrics that excluded Baetidae. Ephemeroptera Richness less Baetidae and % Ephemeroptera less Baetidae had the highest GAMM R^2 values in both seasons. In Spring, when Ephemeroptera were most abundant, non-Baetidae genera had median salt-sensitivity rank of 10.5, compared to median rank of 29 for Baetidae genera.

Our observational findings on the relative insensitivity of Baetids to salinity are in contrast to experimental results of Kefford et al. (2003, 2012) who found Baetids to be less sensitive than other Ephemeroptera found commonly elsewhere in the world (i.e., Australia, France, Israel, and South Africa), but differences in specific taxa studied, biological endpoints, background salinity, ionic matrix, salt exposure regime, and life stage of exposure are among the variety of reasons to account for such contrast. Our results concur with field-based findings of others working in streams of central Appalachia, USA that Ephemeroptera metrics excluding salt-tolerant Baetidae are more responsive than aggregate Ephemeroptera metrics in streams salinized by coal mining (Merriam et al., 2011; Pond et al., 2013).

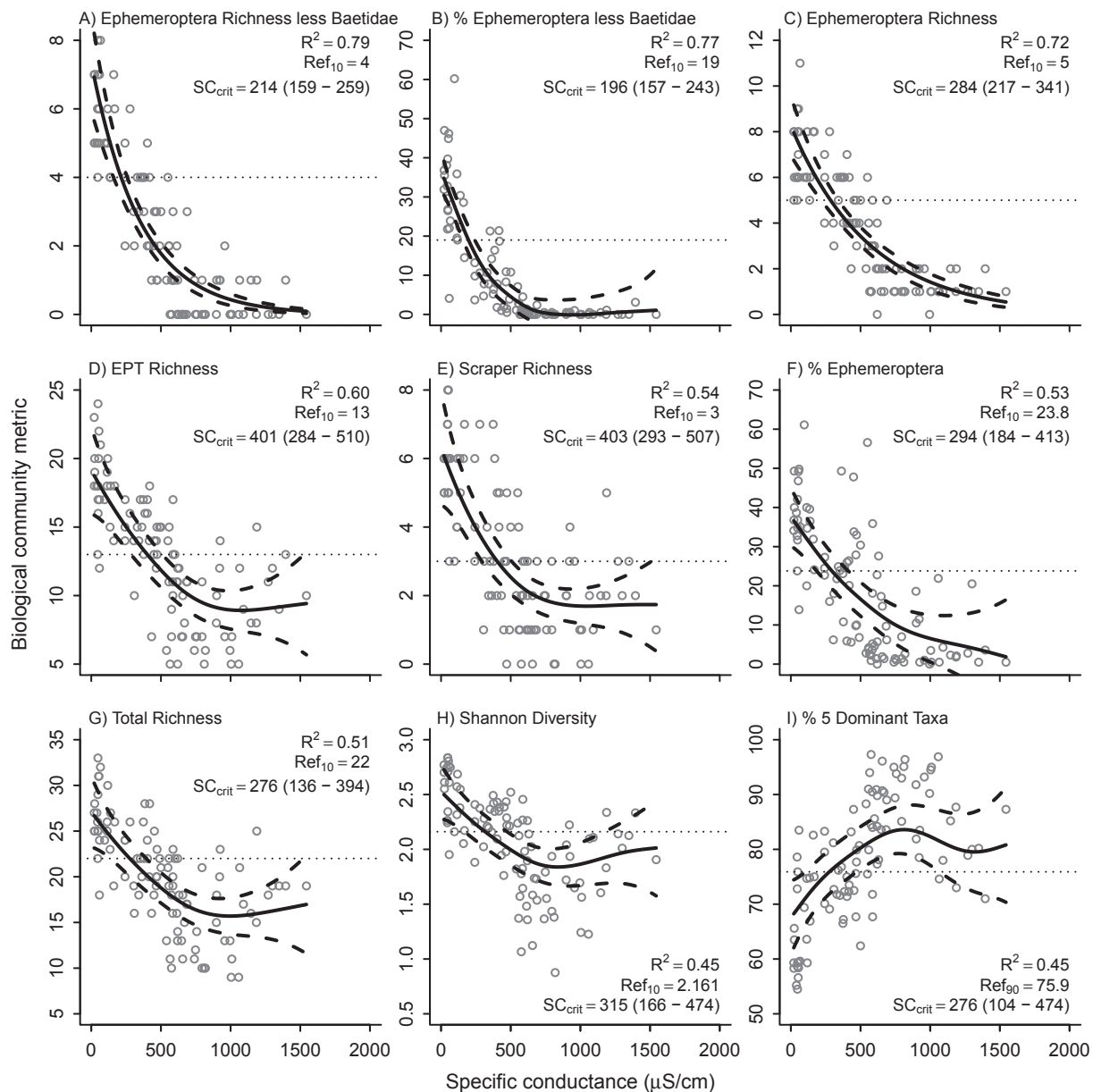


Fig. 3. Generalized additive mixed models of specific conductance and community metrics for Spring. Specific conductance observations (gray circles), with smoothed fit (solid line) and 95% confidence limits (dashed lines). Dotted line is reference threshold (Ref₁₀ or Ref₉₀), the intersection of which by fitted lines denotes critical conductivity value, SC_{crit} (with 95% confidence limits).

Plecoptera, often considered with Ephemeroptera as generally sensitive to perturbation (Barbour et al., 1999), were much less strongly and consistently responsive to SC at the order level, an observation made by others in similar streams (Pond et al., 2008, 2014; Pond, 2010; Merriam et al., 2011). At the genus level, some Plecoptera taxa were relatively sensitive (e.g., *Pteronarcys* in Spring and *Paracapnia* in Fall, Tables 3 and 4), but as a group, Plecoptera metrics were influenced by abundant and tolerant genera *Amphinemura* and *Leuctra* (Table 3), which increased in relative abundance along the SC gradient in Spring, and by *Allocapnia* (Table 4), which exhibited a similar pattern in Fall. Salt-tolerant *Amphinemura* and *Leuctra* can dominate samples in coal-mining-salinized streams (Pond et al., 2008, 2014; Pond, 2012), which could explain why Plecoptera metrics were less sensitive than Ephemeroptera metrics to SC in our study.

Rounding out the trinity of typically pollution-sensitive EPT (Barbour et al., 1999), responses of Trichoptera metrics to SC are similarly masked by a few abundant and tolerant taxa. Despite containing

relatively sensitive genera – *Agapetus* is ranked first in salt sensitivity among Spring taxa (Table 3) and *Pycnopsyche* is ranked ninth among Fall taxa (Table 4) – Trichoptera metrics are driven by genera of the ubiquitous and tolerant family *Hydropsychidae*, with a median genus salt-sensitivity rank of 45 for both seasons (Tables 1 and 2). It is possible that we did not detect a strong salt signal from Trichoptera because of inherent bias of the single-habitat sampling protocol. Trichoptera are very diverse, yet we collected only 26 genera (13 or 14 in a given season) of approximately 150 genera known in North America (Merritt et al., 2008), ostensibly because our focus on erosional habitat (riffle-run complex) does not allow for collection of Trichoptera that inhabit depositional areas in a stream. A broader sampling effort to encompass all habitat types could improve quantification of salinity effects on Trichoptera taxa in salinized streams.

Scraper richness in Spring was the only functional feeding group metric exhibiting a moderate negative association with salinity (Fig. 3E). Scraper taxa found in samples included genera from orders

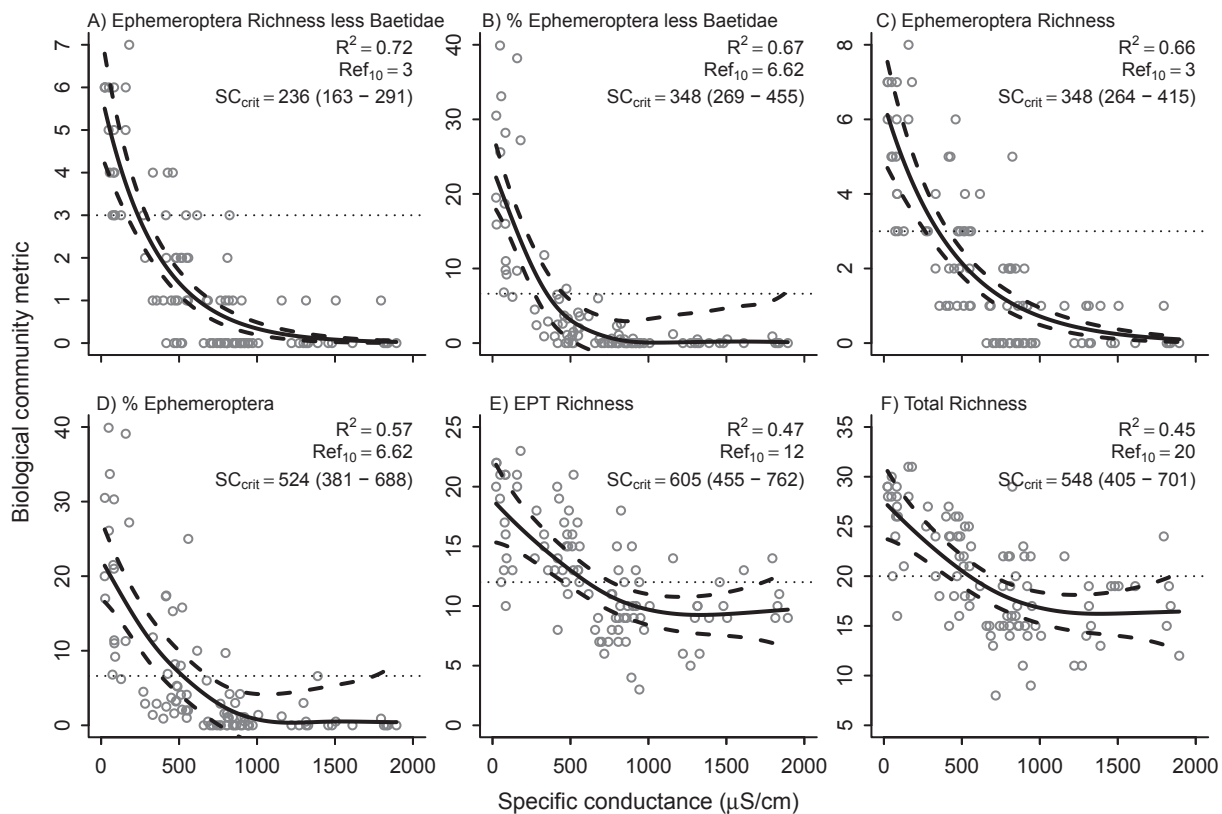


Fig. 4. Generalized additive mixed models of specific conductance and community metrics for Fall. Specific conductance observations (gray circles), with smoothed fit (solid line) and 95% confidence limits (dashed lines). Dotted line is reference threshold (Ref_{10}), the intersection of which by fitted lines denotes critical conductivity value, SC_{crit} (with 95% confidence limits).

Ephemeroptera, Coleoptera, and Trichoptera, which differ in their physiology and life histories but which share a common route of exposure to contaminants: consumption of periphyton (Merritt et al., 2008). Selenium is often elevated along with salinity in streams influenced by coal mining in central Appalachia, where bioaccumulation and trophic transfer of Se is a risk to aquatic life (USEPA, 2011). Therefore, we were concerned that some of the apparent salinity response we observed for scrapers may be attributable to Se toxicity (Conley et al., 2009). Recent research in a subset of our study streams has found that water-column SC can be correlated with Se, which in turn can bioaccumulate in macroinvertebrates (Whitmore, unpublished data), but a clear link between water-column Se and scraper response has not been established. It is possible that excessive Se could cause confounding of salinity-biota models, but only if Se is of sufficient concentration to cause toxicity. However, we do not suspect that Se had appreciable influence on our estimation of SC_{crit} , because the eight Spring samples with Se greater than the U.S. EPA CCC ($3.1 \mu\text{g Se/L}$) had $SC \geq 620 \mu\text{S/cm}$ (range: 620–1092 $\mu\text{S/cm}$), a salinity level above which our modeled scraper response was relatively flat (Fig. 3E). In addition, those samples were similar in scraper richness to other samples within that salinity range from streams with Se less than the CCC.

Mechanisms explaining the unequal field-based salt sensitivity among taxa are a topic of ongoing research, but recent studies suggest that ion toxicity or osmoregulatory stress are likely drivers that induce behavioral drift (Clements and Kotalik, 2016), impede growth and development (Johnson et al., 2015), and/or cause mortality (Kunz et al., 2013). These responses are more pronounced in mayflies, likely because of their phylogenetic and physiological differences. Ephemeroptera evolved from a lineage that diverged from most other aquatic insect orders approximately 410 million years ago (Misof et al., 2014). Differential evolutionary age suggests a unique evolutionary history of mayflies, which may include different osmoregulatory ability (Kefford

et al., 2016; Scheibener et al., 2016), as well as more-frequent molting that may increase susceptibility to stressors (Camp et al., 2014). In addition, there is evidence to suggest that early instars may be more salt-sensitive than later instars (Kefford et al., 2004a, 2007), but recent studies have noted that final instars and/or adult emergence may also be a time of high sensitivity to metals (Schmidt et al., 2013; Wesner et al., 2014) and salts (Soucek and Dickinson, 2015) alike. Additional experimental investigation into the suspected high metabolic costs of osmoregulation and effects of ion mixtures (Kunz et al., 2013), as well as possible behavioral or life-stage-dependent responses (Clements et al., 2013; Clements and Kotalik, 2016) has potential to reveal mechanisms controlling salinity effects to benthic macroinvertebrates.

Identification of taxa-specific relative salt sensitivities enables assessments using metrics that emphasize sensitive taxa and exclude tolerant taxa, which can increase the ability to detect community changes in response to increased salinity (Pond, 2010, 2012; Merriam et al., 2011). Our data support excluding salt-tolerant mayflies and stoneflies from their respective order-level metrics when seeking to detect low-level salinity effects on community structure. Pond et al. (2013) justify removing the tolerant caddisfly *Cheumatopsyche* from the % EPT metric, though other reasonable adjustments of aggregate metrics are possible and could be considered to increase metric sensitivity.

4.2. Critical conductivity levels

Field-based critical conductivity levels are influenced by multiple factors, including biological sample timing, modeling framework, SC predictor, and biological condition target, many of which differ among studies of salinization in streams in the central Appalachian region (Timpano et al., 2015). Therefore, SC_{crit} values should be interpreted in light of those factors, recognizing that they may not be directly comparable across studies or transferrable to novel streams or ecoregions.

Rather than identifying definitive SC levels of concern, our SC_{crit} values provide approximations that contribute to the larger body of evidence that may be useful in developing salinity management policies and procedures. In addition, comparability between our SC_{crit} values and those observed by others validates methods used here, which may be more broadly transferrable than specific SC_{crit} values. Comparisons of SC_{crit} among metrics can be made to assess relative salt-sensitivities of those metrics.

Because it is reasonable to expect that the most sensitive members of the community would respond first to increasing salinity, critical conductivity levels for mayfly metrics indicate the salinity associated with onset of community change when conditions begin to differentiate from reference. Only a few studies of mining-salinized streams in central Appalachia have estimated critical SC levels with emphasis on mayfly taxa, but despite differing methods, those studies found SC_{crit} values similar to ours. An analysis of Spring data indicated that % Ephemeroptera in mining-salinized streams was less than in unsalinized reference streams, with a change point in the range of 124–336 $\mu\text{S}/\text{cm}$ (Pond, 2010), which overlaps the SC_{crit} range of 184–413 $\mu\text{S}/\text{cm}$ we found for % Ephemeroptera. Sampling in Spring, Merriam et al. (2011) observed that Ephemeroptera Richness and % Ephemeroptera less Baetidae were less than reference-stream levels at $SC \geq 168 \mu\text{S}/\text{cm}$. That is near the lower 95% confidence limit of SC_{crit} that we observed for % Ephemeroptera less Baetidae (157–243 $\mu\text{S}/\text{cm}$), but lower than we observed for Ephemeroptera Richness (217–341 $\mu\text{S}/\text{cm}$). Analyzing 50 taxa that declined in abundance with increasing salinity, 17 of which were mayflies, Bernhardt et al. (2012) found the greatest cumulative decline in community diversity occurred from 178–289 $\mu\text{S}/\text{cm}$. That range is comparable to the range we observed for declines in Total Richness (136–394 $\mu\text{S}/\text{cm}$), Ephemeroptera Richness less Baetidae (159–259 $\mu\text{S}/\text{cm}$), Ephemeroptera Richness (217–341 $\mu\text{S}/\text{cm}$), % Ephemeroptera (184–413 $\mu\text{S}/\text{cm}$), and % Ephemeroptera less Baetidae (157–243 $\mu\text{S}/\text{cm}$). The comparability of our SC_{crit} levels with those found by others who have conducted studies in the central Appalachian region indicate that our modeling approach is effective in describing community changes in response to salinization and estimating conductivity levels associated with those changes.

In an examination of community-level responses to experimental salinization mimicking the ion mixtures observed in our study streams, Clements and Kotalik (2016) conducted a 10-day mesocosm experiment of mining-induced salinity effects using insects collected during Fall (October) from an uncontaminated, low-SC stream in Colorado, USA. They reported EC20 values (SC level resulting in 20% difference from controls) for Ephemeroptera abundance ranging from 221–382 $\mu\text{S}/\text{cm}$ for NaHCO_3 exposure and 349–577 $\mu\text{S}/\text{cm}$ for MgSO_4 exposure. Recalculating our % Ephemeroptera SC_{crit} based on a reference threshold of 80% of the mean reference value for % Ephemeroptera in Fall yielded an EC20 of 141 (95% confidence limits = 23–236 $\mu\text{S}/\text{cm}$). That range is similar to the EC20 values Clements and Kotalik (2016) found for Ephemeroptera drift, which was 153–271 $\mu\text{S}/\text{cm}$ in NaHCO_3 and 135–172 $\mu\text{S}/\text{cm}$ in MgSO_4 , with drift rate increasing strongly as SC increased. Findings of those authors suggest that we may have observed results of mayfly behavioral drift in our study, which may be an important, and largely overlooked, mechanism of mayfly population decline in salinized streams.

4.3. Influence of sample timing

Community differences may be influenced by short- and long-term variation in salinity and in community structure, which could confound the salinity-response signal. Natural seasonal variation in sampled benthic macroinvertebrate community composition is expected, as life cycles and phenology vary among taxa (Poff et al., 2006; Merritt et al., 2008). Such seasonal variation of biota can affect assessments of community structure (Linke et al., 1999; Clarke and Hering, 2006; Silveira et al., 2006; Šporka et al., 2006; Álvarez-Cabria et al., 2010; Johnson

et al., 2012). Long-term variation of community composition may arise from trending influences such as climate (Mazor et al., 2009). Intra-seasonal variation is also possible, as relative abundance metrics can increase during a season (Boehme et al., 2016) as seasonal recruitment increases (Mackay, 1992).

The strong seasonal separation of community structure detected here (Fig. 1) allowed for identification of sensitive taxa in both seasons and fitting of strong seasonal regression models for community metrics. However, despite our relatively narrow sampling window each year, we detected a moderate correlation of Julian day with stream scores in ordination space, indicating that sample timing may have influenced measured community structure in addition to salinity. Efforts to minimize variation of intra-seasonal sample timing could minimize error of salinity-biota regression models and increase SC_{crit} precision. Water salinity also exhibits natural seasonal variation of approximately $\pm 20\%$ relative to annual mean levels, with SC in Fall (annual maximum) approximately 50% greater than the SC in Spring (annual minimum; Timpano et al., 2018). That variation could impact quantification of salinity-biota relationships and should be considered when interpreting SC_{crit} values that are specific to narrow water-sampling time windows, such as those generated here.

5. Conclusions

Stark declines in richness and abundance of salt-sensitive taxa such as most Ephemeroptera, combined with simultaneous increases in abundance of a few salt-tolerant taxa in the orders Plecoptera and Trichoptera combine to shape community structure in salinized streams. Hence, although declines in broad community-level metrics such as total richness, EPT richness, evenness, and diversity are observed in response to salinization, they may not be the best indicators of salt stress, as they incorporate both salt-sensitive and salt-tolerant taxa. Rather, our findings suggest that detection of the onset of community change is best accomplished with metrics that focus on genera most sensitive to salinity, such as those in order Ephemeroptera. Although sensitive taxa are not limited to order Ephemeroptera, lower sensitivity and greater variability of other community metrics in response to SC makes them less valuable as bioindicators of salinity effects than Ephemeroptera metrics.

Chronic salinization has persistent effects on community structure over multiple years, but those effects are not uniform among taxa or consistent across seasons. Our findings suggest that effective detection of the onset of community change in salinized streams is best accomplished using Spring data and community metrics that emphasize salt-sensitive Ephemeroptera taxa.

Acknowledgements

This work was funded by the U.S. Office of Surface Mining Reclamation and Enforcement, and the Virginia Department of Mines Minerals and Energy under Cooperative Agreement number S12AC20023. We thank the many landowners, mine operators, and agency personnel who facilitated site selection and access. We also thank two anonymous reviewers for their helpful comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ecolind.2018.04.031>.

References

- Álvarez-Cabria, M., Barquín, J., Juanes, J.A., 2010 Spatial and seasonal variability of macroinvertebrate metrics: Do macroinvertebrate communities track river health? doi: 10.1016/j.ecolind.2009.06.018.
- APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed.

- American Public Health Association, Washington, DC.
- Arle, J., Wagner, F., 2013. Effects of anthropogenic salinisation on the ecological status of macroinvertebrate assemblages in the Werra River (Thuringia, Germany). *Hydrobiologia* 701, 129–148. <http://dx.doi.org/10.1007/s10750-012-1265-z>.
- Barbour, M.T., Gerritsen, J., Snyder, B.D., Stribling, J.B., 1999. Rapid bioassessment protocols for use in streams and Wadeable rivers: periphyton, benthic macroinvertebrates, and fish. Washington, DC.
- Bernhardt, E.S., Lutz, B.D., King, R.S., et al., 2012. How many mountains can we mine? Assessing the regional degradation of central Appalachian rivers by surface coal mining. *Environ. Sci. Technol.* 46, 8115–8122. <http://dx.doi.org/10.1021/es301144q>.
- Boehme, E.A., Zipper, C.E., Schoenholtz, S.H., Soucek, D.J., Timpano, A.J., 2016. Temporal dynamics of benthic macroinvertebrate communities and their response to elevated specific conductance in Appalachian coalfield headwater streams. *Ecol. Ind.* 64, 171–180.
- Camp, A.A., Funk, D.H., Buchwalter, D.B., 2014. A stressful shortness of breath: molting disrupts breathing in the mayfly *Cloeon dipterum*. *Freshwater Sci.* 33, 695–699. <http://dx.doi.org/10.1086/677899>.
- Cañedo-Argüelles, M., Hawkins, C.P., Kefford, B.J., et al., 2016. Saving freshwater from salts: ion-specific standards are needed to protect biodiversity. *Science* 351 (6276), 914–916. <http://dx.doi.org/10.1126/science.123488>.
- Cañedo-Argüelles, M., Kefford, B.J., Piscart, C., et al., 2013. Salinisation of rivers: an urgent ecological issue. *Environ. Pollut.* 173, 157–167. <http://dx.doi.org/10.1016/j.envpol.2012.10.011>.
- Cañedo-Argüelles, M., Grantham, T.E., Perrée, I., et al., 2012. Response of stream invertebrates to short-term salinization: a mesocosm approach. *Environ. Pollut.* 166, 144–151. <http://dx.doi.org/10.1016/j.envpol.2012.03.027>.
- Cao, Y., Hawkins, C., 2011. The comparability of bioassessments: a review of conceptual and methodological issues. *J. North Am. Benthol. Soc.* 30, 680–701. <http://dx.doi.org/10.1899/10-067.1>.
- Chapman, P.M., Bailey, H., Canaria, E., 2000. Toxicity of total dissolved solids associated with two mine effluents to chironomid larvae and early life stages of rainbow trout. *Environ. Toxicol. Chem.* 19, 210–214. <http://dx.doi.org/10.1002/etc.5620190125>.
- Clarke, R.T., Hering, D., 2006. Errors and uncertainty in bioassessment methods - Major results and conclusions from the STAR project and their application using STARBUGS. *Hydrobiologia* 566, 433–439. <http://dx.doi.org/10.1007/s10750-006-0079-2>.
- Clements, W.H., Cadmus, P., Brinkman, S.F., 2013. Responses of aquatic insects to Cu and Zn in stream microcosms: understanding differences between single species tests and field responses. *Environ. Sci. Technol.* 47, 7506–7513. <http://dx.doi.org/10.1021/es401255h>.
- Clements, W.H., Kotalik, C., 2016. Effects of major ions on natural benthic communities: an experimental assessment of the US Environmental Protection Agency aquatic life benchmark for conductivity. *Freshwater Sci.* 35, 126–138. <http://dx.doi.org/10.1086/685085>.
- Conley, J.M., Funk, D.H., Buchwalter, D.B., 2009. Selenium bioaccumulation and maternal transfer in the mayfly *Centroptilum triangulifer* in a life-cycle, periphyton-biofilm trophic assay. *Environ. Sci. Technol.* 43, 7952–7957.
- Cormier, S.M., Suter, G.W., Zheng, L., 2013. Derivation of a benchmark for freshwater ionic strength. *Environ. Toxicol. Chem.* 32, 263–271. <http://dx.doi.org/10.1002/etc.2064>.
- Evans, D.M., Zipper, C.E., Donovan, P.F., Daniels, W.L., 2014. Long-term trends of specific conductance in waters discharged by coal-mine valley fills in central Appalachia, USA. *JAWRA J. Am. Water Resour. Assoc.* 50, 1449–1460. <http://dx.doi.org/10.1111/jawr.12198>.
- García-Criado, F., Tomé, A., Vega, F.J., Antolín, C., 1999. Performance of some diversity and biotic indices in rivers affected by coal mining in northwestern Spain. *Hydrobiologia* 394, 209–217.
- Gerritsen, J., Zheng, L., Burton, J., 2010. Inferring causes of biological impairment in the Clear Fork Watershed, West Virginia. <http://dx.doi.org/10.13140/RG.2.1.5044.4965>.
- Goetsch, P.A., Palmer, C.G., 1997. Salinity tolerances of selected macroinvertebrates of the Sabie River, Kruger National Park, South Africa. *Arch. Environ. Contam. Toxicol.* 32, 32–41. <http://dx.doi.org/10.1007/s002449900152>.
- Green, J., Passmore, M., Childers, H., 2000. A Survey Of The Condition Of Streams In The Primary Region Of Mountaintop Mining/Valley Fill Coal Mining: Mountaintop Mining/Valley Fill Programmatic Environmental Impact Statement. USEPA, Region III, Wheeling.
- Hartman, K.J., Kaller, M.D., Howell, J.W., Sweka, J.A., 2005. How much do valley fills influence headwater streams? *Hydrobiologia* 532, 91–102. <http://dx.doi.org/10.1007/s10750-004-9019-1>.
- Hawkins, C.P., Olson, J.R., Hill, R.A., 2010. The reference condition: predicting benchmarks for ecological and water-quality assessments. *J. North Am. Benthol. Soc.* 29, 312–343. <http://dx.doi.org/10.1899/09-092.1>.
- ILEPA, 2001. Illinois Environmental Protection Agency Rulemaking Proposal, Exhibit S, In the Matter of: Water Quality Triennial Review: Amendments to 35 Adm. Code 302.105, 302.208(e)-(g), 302.504(a), 302.575(d), 309.141(h); and Proposed 35 Ill. Adm. Code 301.267, 301.313, 301.413, 304.120, and 309.157, R02-11 (Rulemaking - Water)(filed with the Pollution Control Board on November 9, 2001).
- Johnson, B.R., Weaver, P.C., Nietch, C.T., et al., 2015. Elevated major ion concentrations inhibit larval mayfly growth and development. *Environ. Toxicol. Chem.* 34, 167–172. <http://dx.doi.org/10.1002/etc.2777>.
- Johnson, R.C., Carreiro, M.M., Jin, H.S., Jack, J.D., 2012. Within-year temporal variation and life-cycle seasonality affect stream macroinvertebrate community structure and biotic metrics. *Ecol. Ind.* 13, 206–214. <http://dx.doi.org/10.1016/j.ecolind.2011.06.004>.
- Kefford, B.J., 1998. The relationship between electrical conductivity and selected macroinvertebrate communities in four river systems of south-west Victoria, Australia. *Int. J. Salt Lake Res.* 7, 153–170.
- Kefford, B.J., Pappas, P.J., Nuggeoda, D., 2003. Relative salinity tolerance of macroinvertebrates from the Barwon River, Victoria, Australia. *Mar. Freshwater Res.* 54, 755–765. <http://dx.doi.org/10.1071/MF02081>.
- Kefford, B., Dalton, A., Palmer, C., Nuggeoda, G., 2004a. The salinity tolerance of eggs and hatchlings of selected macroinvertebrates in Southeast Australia and South Africa. *Hydrobiologia* 517, 179–192.
- Kefford, B.J., Pappas, P.J., Metzeling, L., Nuggeoda, D., 2004b. Do laboratory salinity tolerances of freshwater animals correspond with their field salinity? *Environ. Pollut.* 129, 355–362. <http://dx.doi.org/10.1016/j.envpol.2003.12.005>.
- Kefford, B.J., Hickey, G.L., Gasith, A., Ben-David, E., Dunlop, J.E., Palmer, C.G., Allan, K., Choy, S.C., Piscart, C., 2012. Global scale variation in the salinity sensitivity of riverine macroinvertebrates: Eastern Australia, France, Israel and South Africa. *PLoS One* 7. <http://dx.doi.org/10.1371/journal.pone.0035224>.
- Kefford, B.J., Buchwalter, D., Cañedo-Argüelles, M., et al., 2016. Salinized rivers: degraded systems or new habitats for salt-tolerant faunas? *Biol. Lett.* 12, 1–7. <http://dx.doi.org/10.1098/rsbl.2015.1072>.
- Kefford, B.J., Nuggeoda, D., Zaluzniak, L., et al., 2007. The salinity tolerance of freshwater macroinvertebrate eggs and hatchlings in comparison to their older life-stages: a diversity of responses - The salinity tolerance of freshwater macroinvertebrate eggs and hatchlings. *Aquat. Ecol.* 41, 335–348. <http://dx.doi.org/10.1007/s10452-006-9066-y>.
- Kennedy, A.J., Cherry, D.S., Currie, R.J., 2003. Field and laboratory assessment of a coal processing effluent in the Leading Creek Watershed, Meigs County, Ohio. *Arch. Environ. Contam. Toxicol.* 44, 324–331. <http://dx.doi.org/10.1007/s00244-002-2062-x>.
- Kunz, J.L., Conley, J.M., Buchwalter, D.B., et al., 2013. Use of reconstituted waters to evaluate effects of elevated major ions associated with mountaintop coal mining on freshwater invertebrates. *Environ. Toxicol. Chem.* 32, 2826–2835. <http://dx.doi.org/10.1002/etc.2391>.
- Legendre, P., Legendre, L., 2012. *Numerical Ecology*, third ed. Elsevier B.V, Amsterdam, Netherlands, pp. 1006.
- Linke, S., Bailey, R.C., Schwindt, J., 1999. Temporal variability of stream bioassessments using benthic macroinvertebrates. *Freshwater Biol.* 42, 575–584. <http://dx.doi.org/10.1046/j.1365-2427.1999.00492.x>.
- Mackay, R.J., 1992. Colonization by lotic macroinvertebrates: a review of processes and patterns. *Can. J. Fish. Aquat. Sci.* 49, 617–628. <http://dx.doi.org/10.1139/f92-071>.
- Mazor, R.D., Purcell, A.H., Resh, V.H., 2009. Long-Term Variability in Bioassessments: A Twenty-Year Study from Two Northern California Streams. doi: 10.1007/s00267-009-9294-8.
- McCune, B., Grace, J., Urban, D., 2002. Analysis of ecological communities.
- Merriman, E.R., Petty, J.T., Merovich, G.T., et al., 2011. Additive effects of mining and residential development on stream conditions in a central Appalachian watershed. *J. North Am. Benthol. Soc.* 30, 399–418. <http://dx.doi.org/10.1899/10-079.1>.
- Merritt, R., Cummins, K.W., Berg, M.B. (Eds.), 2008. *Introduction to the Aquatic Insects of North America*, fourth ed. Kendall Hunt Publishing, Dubuque, Iowa.
- Misof, B., Liu, S., Meusemann, K., et al., 2014. Phylogenomics resolves the timing and pattern of insect evolution. *Science* 346, 763–767. <http://dx.doi.org/10.1126/science.1257570>.
- Oksanen, J., Blanchet, F.G., Friendly, M., et al., 2017. *vegan: Community Ecology Package*. R package version 2.4-3. <https://CRAN.R-project.org/package=vegan>.
- Paybins, K.S., Messinger, T., Eychaner, J.H., et al., 2000. Water Quality in the Kanawha–New River Basin West Virginia, Virginia, and North Carolina. 1996–98: U. S. Geological Survey Circular 1204.
- Piscart, C., Moreteau, J.-C., Beisel, J.-N., 2005. Biodiversity and structure of macroinvertebrate communities along a small permanent salinity gradient (Meurthe River, France). *Hydrobiologia*. <http://dx.doi.org/10.1007/s10750-005-4463-0>.
- Poff, N.L., Olden, J.D., Vieira, N.K.M., et al., 2006. Functional trait niches of North American lotic insects: traits-based ecological applications in light of phylogenetic relationships. *J. North Am. Benthol. Soc.* 25, 730–755.
- Pond, G.J., 2004. Effects of Surface Mining And Residential Land Use On Headwater Stream Biotic Integrity In The Eastern Kentucky Coalfield Region. Frankfort, Kentucky. <http://dx.doi.org/10.13140/RG.2.1.1371.0249>.
- Pond, G.J., 2010. Patterns of Ephemeroptera taxa loss in Appalachian headwater streams (Kentucky, USA). *Hydrobiologia* 641, 185–201. <http://dx.doi.org/10.1007/s10750-009-0081-6>.
- Pond, G.J., 2012. Biodiversity loss in Appalachian headwater streams (Kentucky, USA): plecoptera and trichoptera communities. *Hydrobiologia* 679, 97–117. <http://dx.doi.org/10.1007/s10750-011-0858-2>.
- Pond, G.J., Bailey, J.E., Lowman, B.M., Whitman, M.J., 2013. Calibration and validation of a regionally and seasonally stratified macroinvertebrate index for West Virginia Wadeable streams. *Environ. Monit. Assess.* 185, 1515–1540. <http://dx.doi.org/10.1007/s10661-012-2648-3>.
- Pond, G.J., North, S.H., 2013. Application of a benthic observed/expected-type model for assessing Central Appalachian streams influenced by regional stressors in West Virginia and Kentucky. *Environ. Monit. Assess.* 185, 9299–9320. <http://dx.doi.org/10.1007/s10661-013-3253-9>.
- Pond, G.J., Passmore, M.E., Borsuk, F.A., et al., 2008. Downstream effects of mountaintop coal mining: comparing biological conditions using family- and genus-level macroinvertebrate bioassessment tools. *Benthol. Soc.* 27, 717–737. <http://dx.doi.org/10.1899/08-015.1>.
- Pond, G.J., Passmore, M.E., Pointon, N.D., et al., 2014. Long-Term Impacts on Macroinvertebrates Downstream of Reclaimed Mountaintop Mining Valley Fills in Central Appalachia. *Environ. Manage.* 54, 919–933. <http://dx.doi.org/10.1007/>

- s00267-014-0319-6.
- Quinn, G.P., Keough, M.J., 2002. *Experimental Design and Data Analysis for Biologists*. Cambridge University Press, New York.
- Core Team, R., 2017. R: A language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Scheibener, S.A., Richardi, V.S., Buchwalter, D.B., 2016. Comparative sodium transport patterns provide clues for understanding salinity and metal responses in aquatic insects. *Aquat. Toxicol.* 171, 20–29. <http://dx.doi.org/10.1016/j.aquatox.2015.12.006>.
- Schmidt, T.S., Kraus, J.M., Walters, D.M., Wanty, R.B., 2013. Emergence flux declines disproportionately to larval density along a stream metals gradient. *Environ. Sci. Technol.* 47, 8784–8792. <http://dx.doi.org/10.1021/es3051857>.
- Silveira, M.P., Buss, D.F., Nessimian, J.L., Baptista, D.F., 2006. Spatial and temporal distribution of benthic macroinvertebrates in a Southeastern Brazilian river. *Braz. J. Biol.* 66, 623–632. <http://dx.doi.org/10.1590/S1519-69842006000400006>.
- Smith, D.G., 2001. *Pennak's Freshwater Invertebrates of the United States: Porifera to Crustacea*. J. Wiley, New York, New York.
- Soucek, D.J., Dickinson, A., 2015. Full-life chronic toxicity of sodium salts to the mayfly *Neocloeon triangulifer* in tests with laboratory cultured food. *Environ. Toxicol. Chem.* 34, 2126–2137. <http://dx.doi.org/10.1002/etc.3038>.
- Soucek, D.J., Kennedy, A.J., 2005. Effects of hardness, chloride, and acclimation on the acute toxicity of sulfate to freshwater invertebrates. *Environ. Toxicol. Chem.* 24, 1204–1210. <http://dx.doi.org/10.1897/04-142.1>.
- Šporka, F., Vlek, H.E., Bulánková, E., Krno, I., 2006. Influence of seasonal variation on bioassessment of streams using macroinvertebrates. *Hydrobiologia* 566, 543–555. <http://dx.doi.org/10.1007/s10750-006-0073-8>.
- Stewart, K.W., Stark, B., Stanger, J.A., 1993. *Nymphs of North American stonefly genera (Plecoptera)*. University of North Texas Press, Denton, Texas.
- Timpano, A.J., Schoenholtz, S.H., Soucek, D.J., Zipper, C.E., 2015. Salinity as a limiting factor for biological condition in mining-influenced central Appalachian headwater streams. *J. Am. Water Resour. Assoc.* <http://dx.doi.org/10.1111/jawr.12247>.
- Timpano, A.J., Vander Vorste, R., Soucek, D.J., Whitmore, K., Zipper, C.E., Schoenholtz, S.H., 2017. Stream ecosystem response to mining-induced salinization in central Appalachia. Final Performance Report for Cooperative Agreement S15AC20028. U.S. Office of Surface Mining Reclamation and Enforcement. http://www.osmre.gov/programs/TDT/appliedScience/2015VT_SSchoenholtz_StreamEcosystemResponse_FR.pdf.
- Timpano, A.J., Zipper, C.E., Soucek, D.J., Schoenholtz, S.H., 2018. Seasonal pattern of anthropogenic salinization in temperate forested headwater streams. *Water Res.* 133, 8–18. <http://dx.doi.org/10.1016/j.watres.2018.01>.
- USEPA, 2011. *The Effects of Mountaintop Mines and Valley Fills on Aquatic Ecosystems of the Central Appalachian Coalfields*. U.S. Environmental Protection Agency Office of Research and Development, National Center for Environmental Assessment, Washington, DC. EPA/600/R-09/138F.
- USEPA, 2012. *National Recommended Water Quality Criteria*. U.S. Environmental Protection Agency.
- USEPA, 2016. *Aquatic Life Ambient Water Quality Criterion for Selenium – Freshwater 2016*. U.S. Environmental Protection Agency.
- VDEQ, 2008. *Biological Monitoring Program Quality Assurance Project Plan for Wadeable Streams and Rivers*. Virginia Department of Environmental Quality Water Quality Monitoring and Assessment Programs, Richmond, Virginia.
- Wesner, J.S., Kraus, J.M., Schmidt, T.S., et al., 2014. Metamorphosis enhances the effects of metal exposure on the mayfly, *centropilum triangulifer*. *Environ. Sci. Technol.* 48, 10415–10422. <http://dx.doi.org/10.1021/es501914y>.
- Wiggins, G.B., 1996. *Larvae of the North American caddisfly genera (Trichoptera)*. University of Toronto Press, Toronto, Ontario.
- Wood, S., Scheipl, F., 2016. gamm4: Generalized Additive Mixed Models using “mgcv” and “lme4.” R package version 0.2-4. <https://CRAN.R-project.org/package=gamm4>.
- Wright, I.A., Belmer, N., Davies, P.J., 2017. Coal Mine water pollution and ecological impairment of one of Australia's Most “protected” high conservation-value rivers. *Water Air Soil Pollut.* <http://dx.doi.org/10.1007/s11270-017-3278-8>.
- WVDEP, 2015. *Watershed Assessment Branch 2015 Field Sampling Standard Operating Procedures*. West Virginia Department of Environmental Protection Division of Water and Waste Management, Watershed Assessment Branch, Charleston, WV.