

# Periphyton structure and function in constructed headwater streams of the Appalachian coalfield

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**Abstract:** Streams are often constructed on reclaimed coal mines to offset stream losses caused by mining disturbance in the Appalachian coalfield of the Eastern USA. Periphyton is an important component of the organic matter resources in these lotic ecosystems. We compared both short-term (~2 mo) and long-term (9–11 mo) dynamics of periphyton in 8 recently (most <10 y old) constructed, low-order streams in mined areas with that in 4 forested reference streams. We used artificial substrates to quantify periphyton biomass as ash-free dry mass (AFDM), viable algal standing crop as chlorophyll *a* (Chl *a*) concentration, organic matter from senescent autotrophic cells as phaeopigment (phaeo) concentration, and periphyton structure as the autotrophic index (AI) and Chl *a*:phaeo ratio. We compared short-term periphyton accrual rates of AFDM ( $r_{AFDM}$ ) and Chl *a* ( $r_{Chla}$ ) between the stream types. In constructed streams, we also evaluated relationships between environmental factors and both  $r_{Chla}$  and peak algal standing crop. Over the long term, mean AFDM and Chl *a* in constructed streams were ~4 and 2× higher, respectively, than in reference streams. The constructed streams also had greater mean AI values relative to reference streams, indicating lower relative abundances of viable algae in the periphyton. Short-term periphyton structural differences were similar to long-term differences, with AFDM and mean Chl *a* in constructed streams exceeding those of reference streams. Accrual rates were higher in constructed streams during summer and fall seasons, suggesting that higher relative light availability during leaf-on seasons in constructed streams is a primary cause of differences in periphyton production. During fall, warmer temperatures in the constructed streams also appear to have increased algal accrual. The shading of benthic habitats by planting native trees in riparian areas of constructed streams may promote autochthonous organic matter dynamics similar to that of reference streams.

**Key words:** stream restoration, mitigation, coal mine reclamation, benthic algae, biofilm, autochthonous organic matter

Coal has been mined in the central Appalachian region of the USA for more than a century (WVGES 2017), causing significant geomorphic and land-cover change (Hooke 1999, Townsend et al. 2009) that continues today. The intensive disturbance and expansive scale of modern surface mining (Copeland 2015) in high-relief terrain present significant challenges to environmental restoration efforts. High drainage densities make central Appalachian streams particularly susceptible to mining disturbance, and mine spoil disposal in valley fills has buried thousands of km of low-order streams in this region (USEPA 2011). Mining firms construct streams as compensatory mitigation (Clean Water Act, 33 U.S.C. §1251 et seq. (1972); section 404, 33 U.S.C. §1344) with the intent of replacing the structural and

functional attributes of natural streams eliminated by mining, often on the areas disturbed by mining (Fritz et al. 2010, Northington et al. 2011, Petty et al. 2013).

The low-order streams lost to Appalachian surface mining typically occur in forests with dense riparian canopies. Benthic assemblages in forested streams largely depend on allochthonous organic matter (OM) for bioavailable energy because shade inhibits primary production (Cummins 1974, Vannote et al. 1980). However, periphyton is also an important source of OM in Appalachian headwater streams (Honeyfield and Maloney 2015).

The term periphyton refers to a biofilm community of autotrophic algae and heterotrophic bacteria, fungi, and protozoans living on or associated with submerged surfaces,

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DOI: 10.1086/700621. Received 26 October 2017; Accepted 30 July 2018; Published online 18 October 2018.  
Freshwater Science. 2018. 37(4):780–794. © 2018 by The Society for Freshwater Science.

along with the surrounding matrix of extracellular polymers and embedded detritus (Mack et al. 1975, Costerton et al. 1995, Wetzel 2001). Benthic algae are the most ubiquitous primary producers in lotic ecosystems (Biggs 1996), although algal contributions to total OM in small, forested headwaters are typically low (Minshall 1978). Nevertheless, benthic algae are rich in nutrients and fatty acids (Delong and Thorp 2006, Cashman et al. 2013), and periphyton can dominate diets of grazer and filter-feeding functional feeding groups (FFGs) of benthic macroinvertebrates (Mayer and Likens 1987, Torres-Ruiz et al. 2007, Hayden et al. 2016). Benthic algae, therefore, can have a disproportionate effect on consumer biomass (Coffman et al. 1971).

Replacing lost stream ecosystem structures and functions by constructing new streams on mined areas can be challenging because of the nature of Appalachian mining disturbances. Environmental conditions on mined sites often hinder re-establishment of native trees (Burger et al. 2005). Thus, streams on mines often remain without riparian canopies for years after construction, and have low inputs of allochthonous OM (Petty et al. 2013, Krenz et al. 2016). Lack of a riparian canopy increases the intensity of photosynthetically active radiation (PAR) reaching benthic surfaces (Hill and Knight 1988), which can stimulate in-stream primary production and alter thermal regimes (Brown 1969, Lynch et al. 1984, Weatherley and Ormerod 1990). Additionally, many streams constructed for mitigation originate from sediment detention ponds (Krenz et al. 2016), which can accumulate thermal energy and elevate downstream water temperatures above naturally occurring levels (Van Buren et al. 2000, Jones and Hunt 2010). Constructed streams on mined land typically receive waters from upstream mined areas, further altering in-stream environments. The mining-induced changes in water chemistry of these central Appalachian streams, including elevated conductivity, alkalinity, and trace element concentrations, appear to alter aquatic assemblages. These effects have been observed both in reconstructed streams (Merricks et al. 2007, Fritz et al. 2010, Petty et al. 2013) and in natural channels (Hartman et al. 2005, Pond et al. 2008, Timpano et al. 2015). Depressed densities of grazers (Hartman et al. 2005) and reduced abundances of specific grazing taxa (viz., Heptageniidae; Pond 2010) have been reported in mining-influenced saline Appalachian streams. In addition, the use of explosives (Revey 1996) and soil fertilizers (Wilden et al. 2001) in mined watersheds can increase dissolved nitrogen concentrations in streams (Pond et al. 2008, Petty et al. 2013). Therefore, stream ecosystem changes caused by mining may affect periphyton in constructed streams through bottom-up processes (i.e., nutrient enrichment, increased PAR), top-down processes (i.e., release of grazing pressure), or abiotic stressors (i.e., elevated temperature).

The structure and function of periphyton are rarely studied when evaluating stream restoration efforts (Pander and Geist 2013), despite their importance to lotic ecosys-

tems. Furthermore, we are aware of no studies that have assessed how successful constructed streams are in mitigating for the loss of periphyton resources in mined areas. A more complete understanding of periphyton dynamics in constructed streams could better inform management actions designed to mitigate stream loss in mined areas. This 2-y study was designed to answer two questions: 1) Do periphyton structure and function in streams constructed on mined areas differ from those in reference streams? 2) If so, what ecological factors are likely responsible for observed stream-type differences?

## METHODS

### Study design and approach

We studied 8 streams constructed on mined sites and 4 reference streams draining unmined forested watersheds, all in the Appalachian coalfield of Virginia. We compared periphyton dynamics in constructed streams with those in reference streams in 2 ways. First, we used a short-term strategy intended to isolate effects of water chemistry and temperature by limiting confounding effects of top-down effects of herbivory and scour. Second, we used a longer-term strategy to characterize how periphyton dynamics were influenced by all factors present in the streams. We studied each stream over the course of >2 y (July 2010–September 2012). To characterize the environmental setting of each stream, we measured water temperature, stream discharge, and chemistry (biweekly–bimonthly over the study period) and benthic macroinvertebrate communities (4× over the study period). We studied periphyton structure and function in each stream over the course of 2 (short-term) and 9 to 11 m (long-term) by setting out tile arrays to be colonized by periphyton. We deployed the long-term periphyton arrays in the fall season of each year, and we collected periphyton data monthly by removing tiles from the array over the following 9 or 11 months. We conducted the short-term assessments at different times throughout the year to quantify seasonal variability in periphyton dynamics, and collected samples biweekly to monthly. We used the periphyton samples to quantify periphyton structural attributes such as the densities of periphytic chlorophyll *a* (Chl *a*) and biomass and Chl *a*:biomass indicator ratios. We also used these data to quantify periphyton functional attributes such as Chl *a* and biomass accrual rates. We then compared these structural and functional measures between constructed and reference streams to determine if differences in periphyton dynamics existed between stream types.

### Catchment, riparian, and stream characteristics

We selected 8 low-order streams constructed on coal-mined lands and 4 forested reference streams of similar size located on the Pocahontas coal bed in southwestern Virginia within the Appalachian Plateau region (Fig. 1).

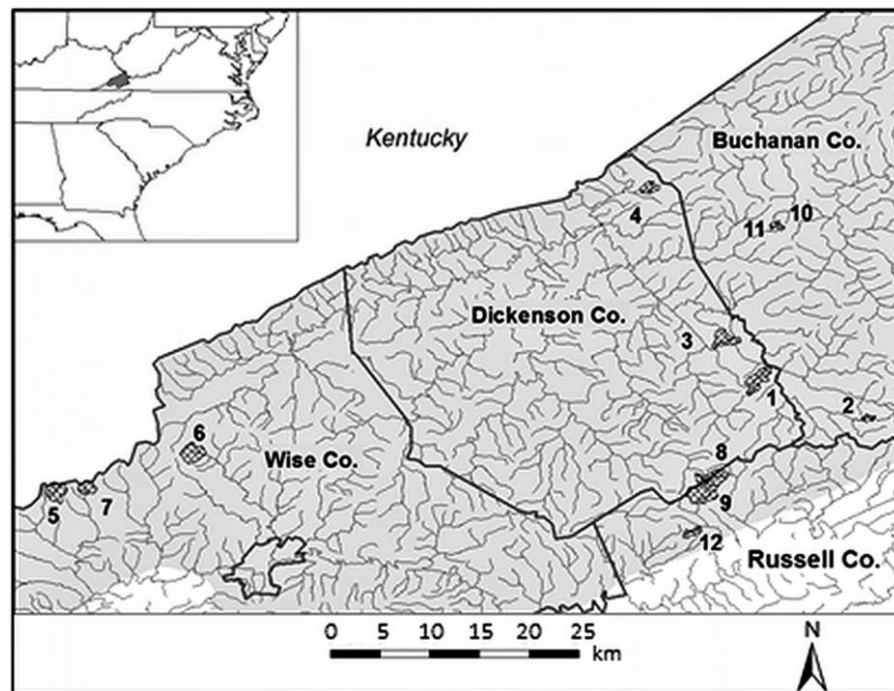


Figure 1. Location of the study streams and their catchments (hatched polygons) in southwestern Virginia. Gray shading indicates Pocahontas coal-bed geology.

All constructed streams were young (mostly <10 y old). Aside from age, we selected stream reaches with similar physical attributes, catchment areas, and other characteristics to facilitate comparisons between constructed and reference streams (Krenz et al. 2016). We selected predominantly forested reference catchments with no residential or commercial development and no current or legacy mines. Each reference stream had  $\geq 50$  m of mature riparian forest on each bank and a USEPA Rapid Bioassessment Protocol (RBP) habitat score (Barbour et al. 1999)  $\geq 175$ . We used ArcGIS 10.1 (ESRI Inc., Redlands, California) to delineate catchments and analyze digital elevation models derived from Virginia Base Mapping Program imagery (<http://www.vita.virginia.gov/isp/>). Catchment- and riparian-level land cover were quantified for each study year with the annual Cropland Data Layer (USDA–National Agricultural Statistics Service). In July 2010, we conducted habitat assessments (Barbour et al. 1999) and physical surveys (Fritz et al. 2006) on one 100-m reach of each stream. We used digital quadrangles (7.5', US Geological Survey) and National Agriculture Imagery Program aerial orthoimagery (US Department of Agriculture–Farm Service Agency) along with personal interviews with mitigation practitioners and mine personnel to determine the presence and age of mining features (e.g., valley fills, sedimentation ponds) (See Supplemental Data, Table S1). Valley fills and sedimentation ponds

were defined as “in-line” if they intersected the current stream channel.

#### Field and laboratory methods

**Physicochemical attributes** We visited study sites biweekly to bimonthly (monthly on average) from Jul 2010 through Sep 2012. Temperature data loggers (HOBO U22; Onset Computer Corp., Bourne, Massachusetts) recorded water temperature at  $\frac{1}{2}$ -hr intervals in the deepest pools of each study reach. We used a spherical densimeter to estimate canopy cover quarterly for the 1<sup>st</sup> y and ~monthly thereafter. We used the velocity-area technique to estimate discharge when water was deep enough to submerge sensors (Marsh–McBirney Flo-Mate; Hach Instruments, Loveland, Colorado).

During each site visit, we used a multi-probe (Hydrolab Quanta; Hach Instruments) to measure dissolved oxygen (DO), temperature ( $^{\circ}\text{C}$ ), specific conductance, and pH in a mixed portion of the water column. We also collected water samples, which we passed through a 0.45- $\mu\text{m}$  filter (Durapore PVDF; EMD Millipore, Darmstadt, Germany) and split into 4 subsamples. One set of subsamples, used for major cation and trace element analyses, was preserved with  $\text{HNO}_3$  at pH <2 prior to transporting to the lab on ice (APHA 2005).

We used flow-injection colorimetry (Seal AutoAnalyzer 3; Seal Analytical, Mequon, Wisconsin) to determine dissolved

ammonium ( $\text{NH}_4^+$ -N), oxidized nitrogen ( $\text{NO}_3 + \text{NO}_2$ -N), and soluble reactive phosphorous (SRP) concentrations. We estimated  $\text{HCO}_3^-$  concentrations from titrimetrically determined alkalinity and instantaneous pH measurements (APHA 2005). Dissolved  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ , Al, Cu, Fe, Mn, Se, and Zn concentrations were determined by inductively coupled plasma optical emissions spectrometry (Varian Vista-MPX CCD Simultaneous ICP-OES; Varian, Walnut Creek, California). Dissolved  $\text{SO}_4^{2-}$  and  $\text{Cl}^-$  concentrations were determined by ion chromatography (DX-500 IC; Dionex Corp., Sunnyvale, California).

**Benthic macroinvertebrates** We used the single-habitat (i.e., riffle-run), Rapid Bioassessment Protocol (Barbour et al. 1999) to sample benthic macroinvertebrates in each stream in early December 2010, late May 2011, mid-October 2011, and mid-April 2012. Two- $\text{m}^2$  samples collected from six 30 s kicks of approximately  $0.3 \text{ m}^2$  each were preserved in 95% ethanol, transported to the lab, and randomly divided into fixed-count subsamples (200 individuals  $\pm$  10%) (VDEQ 2008). We used Merritt et al. (2008) to identify organisms to family level or lower and used the Ecological Data Application System (EDAS, v.3.0, 2000; Tetra Tech, Inc., Owings Mills, Maryland) to calculate taxonomic and functional feeding group (FFG) metrics.

**Periphyton and algae** We obtained periphyton and algal samples from unglazed ceramic tiles placed in each stream (Tuchman and Stevenson 1980, Lamberti and Resh 1983). We deployed tile arrays in early December 2010 and mid-October 2011 to characterize long-term periphyton accrual over 9 (1<sup>st</sup> study year) and 11 (2<sup>nd</sup> study year) months, respectively. Each array consisted of 25  $4.8\text{-cm}^2$  tiles ( $0.57 \text{ cm}$  thick; exposed tile area =  $0.0034 \text{ m}^2$ ) affixed with aquarium silicone that does not inhibit microbial growth to a  $30\text{-cm}^2$  concrete paver ( $5 \text{ cm}$  thick). We deployed 3 tile arrays per stream and placed each array in glides or shallow pools to maintain inundation and minimize deposition, scour, and bed disturbance. All arrays were spaced approximately equidistant ( $\sim 33 \text{ m}$  apart) from one another within each 100-m reach. For the December 2010 arrays, we collected 3 tiles each month from January 2011 through July 2011, and based on the sufficiency of these extract concentrations for quantifying Chl *a*, we reduced collection to 2 tiles from each array in August and September 2011. For the October 2011 arrays, we collected 2 tiles from each array monthly from November 2011 until September 2012.

We characterized short-term periphyton dynamics in each stream by measuring accrual  $5\times$  in each stream for periods of approximately 2 m. Two months allows sufficient time for periphyton development while reducing the likelihood of losses from scouring flows (Aloi 1990). Of the 5 short-term accrual studies, we performed 1 during

the 1<sup>st</sup> study year (winter, December 2010–February 2011), and 4 during the 2<sup>nd</sup> study year (October–December 2011; February–April 2012; April–June 2012; and June–August 2012). Performing the short-term accrual experiments at different times throughout the year allowed us to measure seasonality of short-term periphyton accrual. For 2 of the short-term accrual studies, we used data collected in the first 2 months of the long-term studies (winter, December 2010–February 2011; fall, October–December 2011). For the other 3 short-term accruals, we used tiles glued to  $2.5 \times 30\text{-cm}$  steel strips and affixed to 3 pavers with concrete anchors (Tapcon<sup>®</sup>, ITW Brands, Inc., Schaumburg, Illinois). All arrays were established with the tiles elevated approximately 5 cm above the stream bed to reduce grazing pressures (Lamberti and Resh 1983, Feminella et al. 1989). We did not observe benthic-macroinvertebrate colonization of tiles during the periods of short-term accrual. In the 1<sup>st</sup> year (winter accrual), we collected 3 tiles from each array  $\sim 30$  and  $\sim 60$  d after deployment. In the 2<sup>nd</sup> year (each accrual), we collected 2 tiles from each array at  $\sim 14$ ,  $\sim 25$ – $35$ , and  $\sim 55$ – $75$  d after deployment.

Together, our short- and long-term sampling sequences resulted in 3 replicate measurements per stream-date combination for both long- and short-term analyses. We handled tiles in the same manner for both collection types. After we removed the tiles from the array, we lightly rinsed them in a nonturbulent section of the stream to remove loose allochthonous OM and sediment. We then put the tiles in Ziploc<sup>™</sup> bags that contained a small amount of stream water and transported them to the laboratory on ice for periphyton analyses.

We used AFDM to quantify total periphyton biomass, phaeopigments (phaeo) to quantify OM from senescent autotrophic cells within the biofilm, and phaeopigment-corrected Chl *a* to quantify the viable autotrophic component of the periphyton. We first scraped periphyton from the tiles under low light conditions. We then filtered the resulting periphyton slurry onto preweighed and combusted ( $550^\circ\text{C}$ ) glass fiber filters (F93447VOL; Environmental Express, Charleston, South Carolina). We bisected each filter and used standard methods (APHA 2005) to determine AFDM from  $\frac{1}{2}$  of each filter. We used the other filter  $\frac{1}{2}$  to extract phaeopigment and Chl *a* with 96% ethanol immediately following filtration. Extractions at room temperature lasted for 3–12 h in centrifuge tubes wrapped in foil to exclude light. We then removed the filter and stored the extract at  $-20^\circ\text{C}$  until we determined phaeopigment and phaeopigment-corrected Chl *a* concentrations by monochromatic spectrophotometry (Wasmund et al. 2006, Niederlehner 2010). We adjusted for interference between phaeopigments and Chl *a*, which absorb light at similar wavelengths, by measuring absorbance at 664 and 665 nm before and after acidification (0.1N HCl) of the sample (Lorenzen 1967, APHA 2005, Niederlehner 2010).

We also measured absorbance at 750 nm to correct for turbidity.

### Periphyton metric calculations

**Structural metrics** We calculated mean values of total periphyton standing crops (g AFDM/m<sup>2</sup>), benthic algae (mg Chl *a*/m<sup>2</sup>), and senescent autotrophic material (mg phaeo/m<sup>2</sup>) from the 3 replicate tile arrays collected at each combination of stream and collection date for all long-term and short-term exposures. For long-term exposures, we also calculated mean Chl *a*:phaeo ratios (Tett et al. 1978) and AI values as AFDM/Chl *a* (Weber 1973) from monthly estimates. We excluded stream type–date combinations with periphyton densities  $\leq 2$  g AFDM/m<sup>2</sup> from further analyses, because sparse periphyton standing crops can result in spuriously high or low AI values (Biggs and Kilroy 2000). We analyzed AFDM (long-term and short-term), Chl *a* (long-term and short-term), and AI (long-term) data as temporal sequences, as further described below.

**Functional metrics** We used the equation:

$$N_t/N_0 = e^{rt}, \quad (\text{Eq. 1})$$

to estimate instantaneous accrual rates for periphyton ( $r_{\text{AFDM}}$ ) and algae ( $r_{\text{Chla}}$ ), where  $N_t$  and  $N_0$  are AFDM or Chl *a* standing crops at time  $t$  and at deployment (time 0), respectively (Munn et al. 1989). We used a non-additive analysis of covariance (ANCOVA) model (i.e., including interaction) with dummy coding (Kleinbaum et al. 1988) to estimate periphyton accrual rates for each season. We regressed  $\ln(N_t + 1)$ -transformed concentrations on exposure length expressed in both days (d) and degree-days ( $[N_t + 1]$ -transformation precludes denominators of 0 at  $t = 0$ ). We tested linear slope coefficients representing accrual rates ( $r_{\text{AFDM}}$  or  $r_{\text{Chla}}$ ) from the original exponential model against the null hypothesis that  $r = 0$ , and subsequently, we used Bonferroni-adjusted simple contrasts to compare the accrual rate of each individual constructed stream to the mean reference-stream rate during each season. For accrual rate calculations, we treated data from individual arrays as statistical replicates (i.e., data not pooled), and excluded successive measurements beyond peak biomass (Biggs and Kilroy 2000).

### Statistical analyses

**Short-term differences in periphyton between constructed and reference streams** For the short-term accrual data, we used multivariate repeated-measures analysis of variance (rmANOVA) with restricted maximum likelihood estimates of means (PROC MIXED; SAS 9.4; SAS Institute, Cary, North Carolina) to determine if stream types differed

in peak (maximum) values of accumulated AFDM<sub>max</sub>, Chl  $a_{\text{max}}$ , phaeo<sub>max</sub>,  $r_{\text{AFDM}}$ , and  $r_{\text{Chla}}$ . We further examined statistically significant interactions between stream type and accrual period with Tukey's Honestly Significant Difference (HSD) test for multiple comparisons among seasonal stream-type means. To determine if mean AFDM<sub>max</sub> and Chl  $a_{\text{max}}$  differed between stream types on the final collection date of each accrual period, we substituted Bonferroni-adjusted simple contrasts for omnibus main-effects tests (Maxwell and Delaney 2004). We also used the Bonferroni correction in the nonadditive ANCOVA model (see 'Functional metrics' above) to compare accrual rates of each constructed stream to the mean reference-stream accrual rate for each accrual period. These comparisons are presented only when the interaction (stream type  $\times$  accrual period) in group-wise comparisons was significant and only during accrual periods when differences between stream type were detected by multiple comparison tests. All short-term results are presented by season.

### Long-term differences in periphyton between constructed and reference streams

For long-term accrual data, we also used rmANOVA to determine if differences occurred between stream types in mean AFDM, Chl *a*, phaeo standing crops; AI; and Chl *a*:phaeo. We used the lowest Akaike's information criterion (AIC) score to select appropriate covariance structures for each variable tested (Guo and Hipp 2004). We used Levene's test of homoscedasticity, the Ryan–Joiner test of normality, normal quantile plots, and histograms to assess if data met assumptions for parametric models (Minitab, version 17.1; Minitab Inc., State College, Pennsylvania). We used the nonparametric van Elteren test statistic ( $W'$ ) with study year as the blocking factor (PROC FREQ; SAS version 9.4) to determine if selected physiochemical (daily average and minimum daily temperatures, canopy cover, major ion and NO<sub>3</sub>+NO<sub>2</sub>-N concentrations, water specific conductance, and pH) and macroinvertebrate (relative abundance of scraper–grazers) variables differed between stream types.

### Factors associated with algal structure and function

For the short-term accrual data, we used Spearman rank correlation analyses to examine if  $r_{\text{Chla}}$  and Chl  $a_{\text{max}}$  were associated with measured environmental variables in constructed streams. We used Spearman rank correlations to allow consistent analyses across the range of variables of interest. We conducted these correlation analyses only for short-term accrual periods where algal accrual rates differed significantly by stream type. The correlation analyses were based on seasonal means from each of the 8 constructed streams. In addition, we also included study-year means for benthic macroinvertebrate, land-cover variables, stream habitat, and catchment characteristics (e.g., pond

and valley fill cover). These correlation analyses were exploratory. Results are reported only where correlation coefficients are  $\geq 0.71$ , and we use the strengths of these correlations to identify potential controls on dependent variables in light of our results and observations from other analyses.

## RESULTS

### Environmental and macroinvertebrate structure

Constructed and reference streams differed in several physiochemical and biological attributes over the course of the study (Table 1). First, constructed streams were significantly warmer and  $\text{NO}_3 + \text{NO}_2\text{-N}$ -enriched relative to reference streams. Mean annual  $\text{NO}_3 + \text{NO}_2\text{-N}$  concentrations in constructed streams (2.7 and 1.6 mg/L as N, in years 1 and 2, respectively) were 4 to 5 $\times$  greater than in reference streams (0.4 and 0.5 mg/L as N, respectively) in each year. Furthermore,  $\text{NO}_3 + \text{NO}_2\text{-N}$  concentrations were elevated in constructed streams during all seasons relative to reference streams for both study years. However, the  $\text{NO}_3 + \text{NO}_2\text{-N}$  disparities between stream types were consistently smallest during summer seasons (1<sup>st</sup> summer difference, 1.85 mg N/L; 2<sup>nd</sup> summer difference, 0.98 mg N/L). Additionally, concentrations of all major ions except  $\text{Cl}^-$ , specific conductance, and pH were higher in constructed streams than in reference streams (see Krenz et al. 2016). SRP concentrations were below the detection limit (7  $\mu\text{g P/L}$ ) for 100% of samples, and  $\text{NH}_4\text{-N}$  concentrations were below the detection limit (10  $\mu\text{g N/L}$ ) for 78 and 89% of constructed- and reference-stream samples, respectively. Therefore, SRP and  $\text{NH}_4\text{-N}$  concentrations are not reported. Next, in both study years, the average daily mean temperatures in constructed streams exceeded those of reference streams by  $\geq 0.7^\circ\text{C}$ . Reference-stream mean canopy cover was  $\geq 3\times$  that of constructed streams. Finally, we observed higher proportions of scraper-grazer taxa and greater canopy cover in reference than in constructed streams.

### Long-term periphyton structure

Constructed streams had higher mean AFDM, Chl *a*, and phaeo standing crops than reference streams, with mean values  $\sim 4$ , 2, and 5 $\times$  greater than reference levels, respectively (Table 2). The monthly mean AFDM values in constructed streams exceeded those of reference streams for all accrual periods, particularly in summer (June–August, both study years) (Fig. 2A). Similarly, differences in Chl *a* standing crops between stream types were greatest during the summer months (both study years) (Fig. 2B). Mean monthly Chl *a* standing crops in reference streams exceeded those in constructed streams in March 2012 (8.3 vs 5.8 mg/m<sup>2</sup>) and April 2012 (11.2 versus 7.9 mg/m<sup>2</sup>).

Viable algae were less prevalent in the total periphyton biomass in constructed streams than reference streams (Table 2), despite the higher Chl *a* concentrations in constructed streams. When periphyton was dense enough to quantify AI (i.e.,  $>2 \text{ g AFDM/m}^2$ ), monthly mean AI in reference streams (508) was consistently lower than that of constructed streams (1332) (Table 2, Fig. 2C). Reference stream AI mean values were below 600 every month but June 2011, whereas constructed stream mean AI values exceeded 900 during every month but September 2011. Based on Chl *a*:phaeo ratios, viable algae were 1.4 and 2.5 $\times$  more abundant than senescent autotrophic cells in constructed streams and reference streams, respectively (Table 2). Regardless of stream type, mean AI values and Chl *a*:phaeo ratios were significantly higher in the 1<sup>st</sup> year than the 2<sup>nd</sup> year.

### Short-term periphyton structure

In the short-term studies, peak  $\text{AFDM}_{\text{max}}$ , Chl  $a_{\text{max}}$ , and phaeo<sub>max</sub> standing crops in constructed streams were approximately 4, 3, and 4 $\times$  greater than those in reference streams, respectively (Table 3).  $\text{AFDM}_{\text{max}}$  and Chl  $a_{\text{max}}$  standing crops differed with season in both stream types, but there was a significant interaction between stream type and specific accrual period for peak Chl *a*, which showed

Table 1. Annual means ( $\pm 1$  SE) for selected structural variables important to regulating periphyton biomass and productivity in constructed and reference streams.<sup>a</sup> Avg = average. Min = minimum.

Structural variable	1st year		2nd year		$W'$ ( $p$ -value) <sup>b</sup>
	Constructed	Reference	Constructed	Reference	
Avg. daily mean temp. ( $^\circ\text{C}$ )	12.1 $\pm$ 0.3	11.2 $\pm$ 0.1	13.1 $\pm$ 0.3	12.4 $\pm$ 0.1	5.21 (0.023)
Min. daily mean temp. ( $^\circ\text{C}$ )	1.7 $\pm$ 0.4	0.2 $\pm$ 0.2	3.5 $\pm$ 0.5	1.2 $\pm$ 0.4	12.15 (0.001)
Canopy cover (%)	25 $\pm$ 7	79 $\pm$ 2	21 $\pm$ 5	75 $\pm$ 1	14.77 (<0.001)
$\text{NO}_3 + \text{NO}_2\text{-N}$ (mg N/L)	2.7 $\pm$ 1.0	0.5 $\pm$ 0.1	1.6 $\pm$ 0.6	0.4 $\pm$ 0.1	6.68 (0.010)
Scraper-grazers (%)	5 $\pm$ 2	15 $\pm$ 2	9 $\pm$ 3	13 $\pm$ 1	6.99 (0.008)

<sup>a</sup> For complete listing of structural variables that differed between stream types, see Krenz (2015).

<sup>b</sup> van Elteren's test ( $W'$  [ $p$ -value]) comparing stream-type means blocking for year.

Table 2. Periphyton biomass (AFDM), algal standing crop (Chl *a*), senescent autotrophic organic matter (phaeo), and 2 indicator ratio (AI, Chl *a*:phaeo) means ( $\pm 1$  SE) by stream type and long-term exposure period. *F*-ratios are main-effects tests from repeated measures ANOVA (rmANOVA). Interactions were not significant. \* =  $p \leq 0.05$ , \*\* =  $p \leq 0.005$ , \*\*\* =  $p \leq 0.001$ .

Yearly variable	Stream Type		<i>F</i>	Exposure Period		<i>F</i>
	Constructed ( <i>n</i> = 8)	Reference ( <i>n</i> = 4)		1st year ( <i>n</i> = 12)	2nd year ( <i>n</i> = 12)	
Areal densities						
AFDM (g/m <sup>2</sup> )	10.21 $\pm$ 1.45	2.52 $\pm$ 0.56	23.78***	8.19 $\pm$ 1.65	7.10 $\pm$ 1.88	1.88
Chl <i>a</i> (mg/m <sup>2</sup> )	8.04 $\pm$ 0.91	4.32 $\pm$ 0.48	9.82*	6.63 $\pm$ 0.65	6.97 $\pm$ 1.31	0.08
phaeo (mg/m <sup>2</sup> )	9.79 $\pm$ 1.96	1.82 $\pm$ 0.33	19.66**	5.73 $\pm$ 1.35	8.54 $\pm$ 2.72	2.21
Indicator ratios						
AI	1,332 $\pm$ 126	508 $\pm$ 41	25.33***	1,167 $\pm$ 179	948 $\pm$ 151	8.19*
Chl <i>a</i> :phaeo	1.41 $\pm$ 0.11	2.54 $\pm$ 0.18	25.63***	2.04 $\pm$ 0.21	1.53 $\pm$ 0.18	24.11***

that seasonal patterns in algal standing crops differed between stream types. In particular, the differences in Chl *a*<sub>max</sub> values between constructed and reference streams were higher in fall and summer (Fig. 3A).

The difference between stream types in peak short-term accrual of periphyton biomass also varied significantly by season (Fig. 4A–). On the final collection date of fall 2011, mean AFDM was 5.0 g/m<sup>2</sup> higher and Chl *a* was 7.4 mg/m<sup>2</sup> higher in constructed streams than in reference streams. Mean AFDM and Chl *a* concentration were also significantly higher in constructed streams on the final collection dates of late spring 2012 (AFDM difference = 5.2 g/m<sup>2</sup>, Chl *a* difference = 10.4 mg/m<sup>2</sup>) and summer 2012 (AFDM difference = 10.0 g/m<sup>2</sup>, Chl *a* difference = 14.3 mg/m<sup>2</sup>). However, mean AFDM and Chl *a* concentrations did not differ significantly between stream types in either winter or early spring.

### Short-term periphyton function

Algae accrued more rapidly in constructed streams than in reference streams over the short-term accrual periods (Table 3), and accrual rates differed between stream types and among seasons (Fig. 3B, C). Significant statistical interactions between stream type and accrual period for  $r_{\text{Chl}a/\text{day}}$ ,  $r_{\text{Chl}a/\text{degree-day}}$ , and  $r_{\text{AFDM}/\text{degree-day}}$  indicated that seasonal patterns of accrual differed between reference and constructed streams. The Tukey's HSD test showed that mean  $r_{\text{Chl}a/\text{day}}$  was significantly higher in constructed streams than in reference streams for only fall and summer (Fig. 3B), and  $r_{\text{Chl}a/\text{degree-day}}$  was higher in constructed than in reference streams only in summer (Fig. 3C). However,  $r_{\text{AFDM}/\text{degree-day}}$  did not differ between stream types in any season (results not shown).

In several constructed streams, mean summer and fall  $r_{\text{Chl}a/\text{day}}$  and  $r_{\text{Chl}a/\text{degree-day}}$  differed from reference stream values (Table S2). After we corrected the data for temperature, the differences in mean  $r_{\text{Chl}a}$  between stream types re-

mained significant for summer but not for fall (Fig. 3C). In summer,  $r_{\text{Chl}a/\text{day}}$  values for 5 constructed streams and  $r_{\text{Chl}a/\text{degree-day}}$  values for 6 constructed streams were higher than the reference mean, whereas the  $r_{\text{Chl}a/\text{day}}$  and  $r_{\text{Chl}a/\text{degree-day}}$  values were similar to reference means in both summer and fall only for 1 constructed stream (STO) (Table S2). Individual among-stream comparisons are not presented for other rate–accrual period combinations,

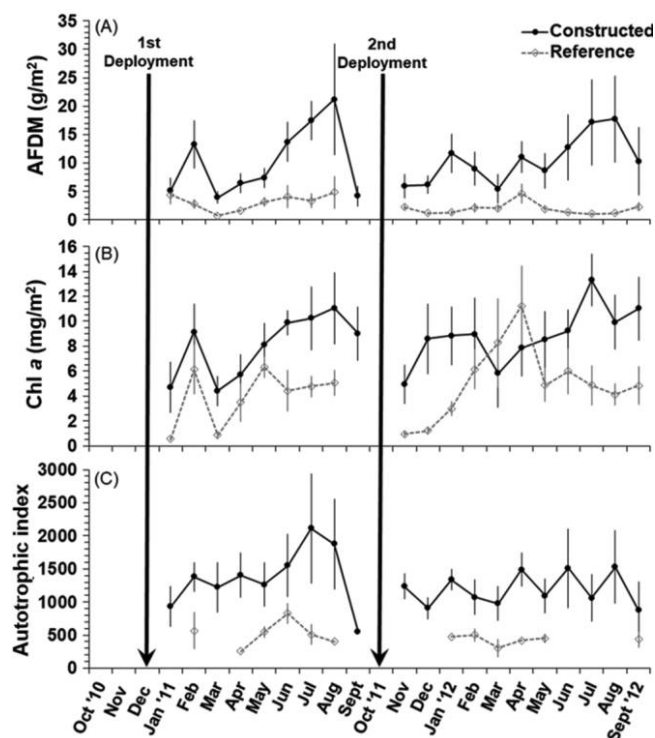


Figure 2. Mean ( $\pm$ SE) periphyton biomass as AFDM (A), benthic algal standing crop as Chl *a* (B), and autotrophic index values (C) over long-term exposures for constructed and reference streams. Gaps indicate that periphyton biomass was  $\leq 2$  g AFDM/m<sup>2</sup> for all reference streams during that month.

Table 3. Short-term mean ( $\pm 1$  SE) accrual rates ( $r_{AFDM}$  and  $r_{Chla}$ ) and peak densities of periphyton (AFDM), algae (Chl *a*), and senescent autotrophic organic matter (phaeo) by stream type and accrual period. *F*-ratios are main effects tests from repeated measures analysis of variance (rmANOVA). See Krenz (2015) for further details on covariance structures and transformations. \* =  $p \leq 0.05$ , \*\* =  $p \leq 0.005$ , and \*\*\* =  $p \leq 0.001$ .

Accrual Period Variables	Stream type				Short-term accrual period				<i>F</i>	
	Constructed ( <i>n</i> = 8)	Reference ( <i>n</i> = 4)	Fall ( <i>n</i> = 12)	Winter ( <i>n</i> = 12)	Early Spring ( <i>n</i> = 12)	Late Spring ( <i>n</i> = 12)	Summer ( <i>n</i> = 12)			
<b>Peak areal densities</b>										
AFDM <sub>max</sub> (g/m <sup>2</sup> )	9.14 ± 1.37	2.49 ± 0.45	5.91 ± 1.52	10.93 ± 2.93	4.99 ± 1.40	5.06 ± 1.43	7.74 ± 3.20	3.46*		
Chl <i>a</i> <sub>max</sub> (mg/m <sup>2</sup> ) <sup>a</sup>	12.91 ± 1.63	4.99 ± 1.24	6.37 ± 2.07	8.93 ± 1.76	12.68 ± 3.37	11.33 ± 3.41	12.05 ± 3.09	3.84*		
phaeo <sub>max</sub> (mg/m <sup>2</sup> )	11.64 ± 2.23	2.60 ± 0.59	7.87 ± 4.02	9.67 ± 4.19	9.9 ± 4.15	7.44 ± 2.25	8.26 ± 3.4	1.73		
<b>Accrual rates</b>										
$r_{AFDM}$ (/d)	0.184 ± 0.014	0.163 ± 0.019	0.224 ± 0.024	0.194 ± 0.023	0.141 ± 0.017	0.153 ± 0.029	0.174 ± 0.030	2.69*		
$r_{AFDM}$ (/degree-day) <sup>a</sup>	0.0196 ± 0.0022	0.0281 ± 0.0075	0.0243 ± 0.0026	0.0549 ± 0.0084	0.0136 ± 0.0027	0.0102 ± 0.0024	0.0091 ± 0.0015	38.92***		
$r_{Chla}$ (/d) <sup>a</sup>	0.046 ± 0.004	0.025 ± 0.003	0.029 ± 0.005	0.044 ± 0.008	0.036 ± 0.004	0.036 ± 0.007	0.051 ± 0.010	3.16*		
$r_{Chla}$ (/degree-day) <sup>a</sup>	0.0049 ± 0.0007	0.0041 ± 0.0011	0.0030 ± 0.0005	0.0115 ± 0.0015	0.0033 ± 0.0004	0.0024 ± 0.0006	0.0027 ± 0.0006	22.19***		

<sup>a</sup> Significant interaction of main effects (stream type × accrual period) in rmANOVA.



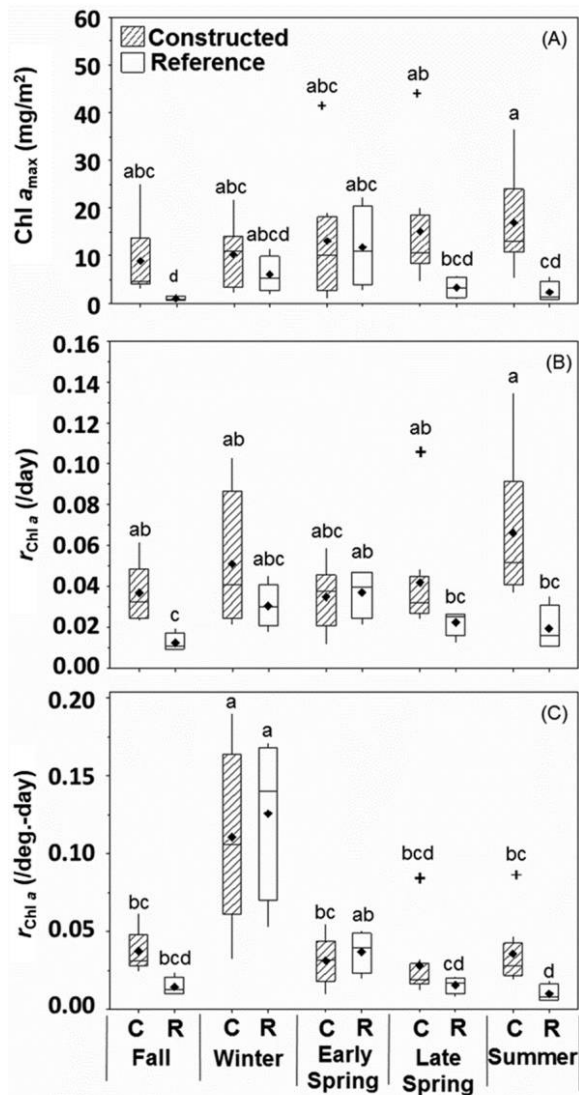


Figure 3. Box-and-whisker plots showing peak algal standing crop ( $\text{Chl } a_{\max}$ ) (A) and algal accrual rates per day (B) and per degree-day (C) over short-term accrual periods in constructed and reference streams. Lines within boxes are medians, closed diamonds are means, box ends are 1<sup>st</sup> and 3<sup>rd</sup> quartiles, whiskers are  $1.5 \times$  the interquartile range, and '+' are outliers. Box plots sharing a lowercase letter are not significantly different based on Tukey's Honestly Significant Difference (HSD;  $p > 0.05$ ) tests following significant interaction ( $F_{4,40} = 5.70$ ,  $p = 0.001$  for [A]; and  $F_{4,40} \geq 4.90$ ;  $p < 0.003$  for [B] and [C]).

because interactions were not significant in group-wise comparisons (Table 3), or multiple comparison tests did not reveal intra-seasonal differences between stream types (Fig. 3B, C).

#### Environmental associations with short-term algal accrual and standing crop

Among constructed streams,  $r_{\text{Chla}}/\text{day}$  and  $\text{Chl } a_{\max}$  were associated with habitat and other physical metrics,

benthic macroinvertebrate metrics, and water temperature metrics in either fall, summer, or both (Table 4). In fall, both  $r_{\text{Chla}}/\text{day}$  and  $\text{Chl } a_{\max}$  were associated positively with minimum daily mean water temperature and relative abundance of chironomids, but were associated negatively with water temperature variability (coefficient of variation), mean baseflow wetted depth, and bankfull depth. Higher  $\text{Chl } a_{\max}$  values in fall were also associated positively with dissolved  $\text{HCO}_3^-$  concentrations and median water temperature, whereas  $r_{\text{Chla}}/\text{day}$  was associated positively with mean particle size ( $D_{50}$ ) of stream sediment.

In summer,  $r_{\text{Chla}}/\text{day}$  and  $\text{Chl } a_{\max}$  in constructed streams were associated with at least 1 physical and 1 benthic macroinvertebrate metric, but not with the same metrics (Table 4). Summer  $r_{\text{Chla}}/\text{day}$  was associated positively with stream slope, coverage of the catchment by in-line valley fills, and relative abundance of the top 2 dominant macroinvertebrate taxa. Summer  $r_{\text{Chla}}/\text{day}$  was associated negatively with bank stability and relative abundance of macroinvertebrate predators.  $\text{Chl } a_{\max}$  was associated positively with minimum daily mean temperature in fall and chironomid relative abundance in summer and fall.  $\text{Chl } a_{\max}$  was associated negatively with minimum daily mean temperature in summer and bankfull depth in both summer and fall. In both summer and fall,  $\text{Chl } a/\text{degree-day}$  was correlated strongly with  $r_{\text{Chla}}/\text{day}$  ( $\rho > 0.92$ , results not shown), so we only discuss correlations with  $r_{\text{Chla}}/\text{day}$  here.

## DISCUSSION

Biomass and accrual of periphyton and benthic algae are often governed by interactive effects of top-down and bottom-up controls (Hill and Harvey 1990, Winterbourn 1990, Rosemond 1993, Hill et al. 1995, Rosemond et al. 2000), but the relative importance of these factors may vary seasonally (Rosemond 1993, Rosemond et al. 2000). However, confounding effects such as scouring flows (Stevenson 1983, Grimm and Fisher 1989) and temperature (Phinney and McIntire 1965, McIntire 1968) can co-vary with both top-down and bottom-up mechanisms, respectively. Here, we found that light and temperature both drive differences in periphyton among stream types, but that the importance of these factors changes throughout the year. Our findings are based on measured values for periphyton structural measures, which were consistent with other scientific literature (Table S3). These findings suggest that intentional establishment of riparian canopy during stream construction would accelerate restoration of periphyton structure and function. Such practice would be consistent with recommendations based on other studies in this region (Krenz et al. 2016, Zipper et al. 2018), and are in agreement with more general stream restoration best practices (e.g., Scarsbrook and Halliday 1999, Kiffney et al. 2003,

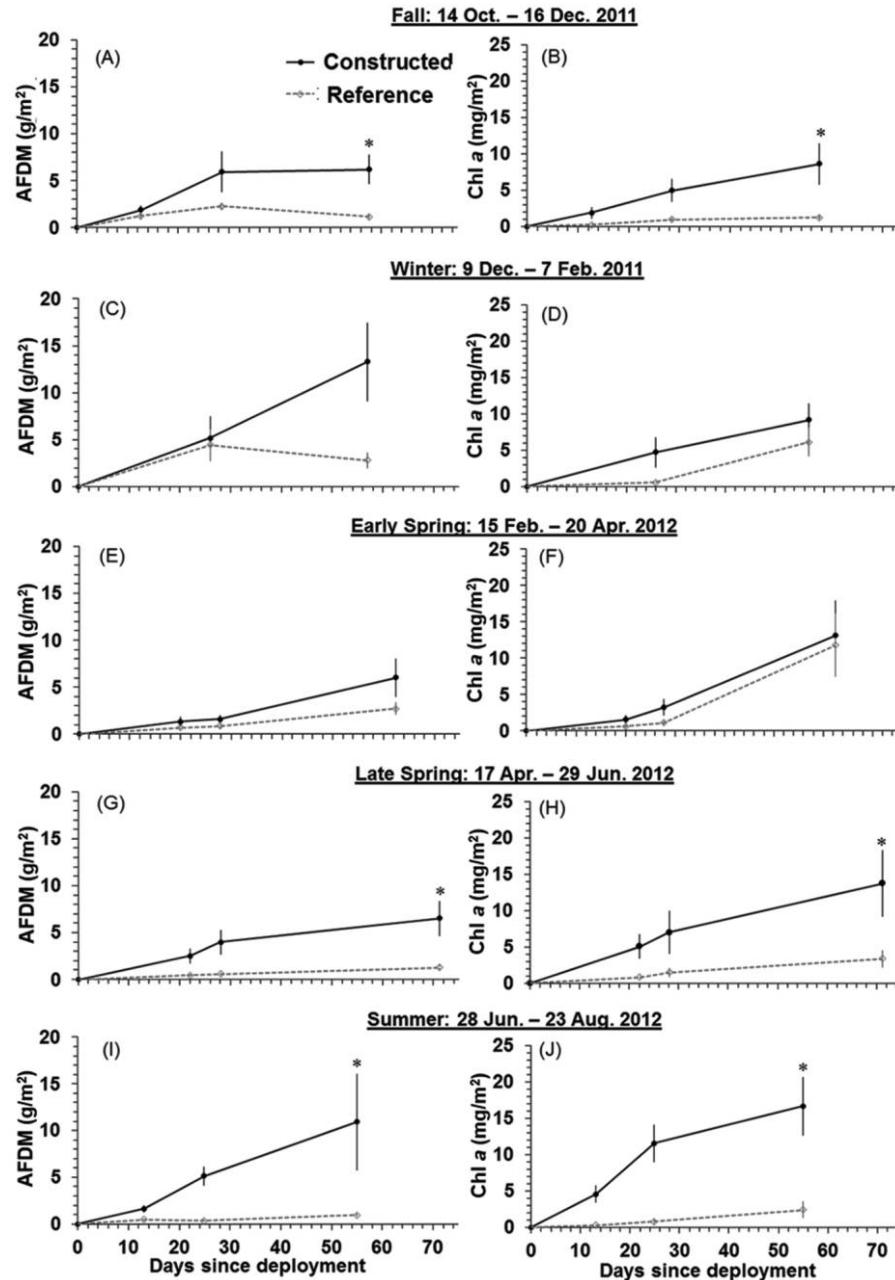


Figure 4. Accrual of periphyton biomass as AFDM (A, C, E, G, I) and algal standing crop as Chl *a* (B, D, F, H, J) over short-term accrual periods for constructed and reference streams. \* = standing crops on the final date (55–75 days) with a significant difference between stream types (Bonferroni-adjusted,  $\alpha = 0.01$ ). Vertical axes are identical among accrual periods for both AFDM and Chl *a*.

Bernhardt et al. 2007, Kaase and Katz 2012, Riley and Dodds 2012, Palmer et al. 2014).

Our results suggest PAR (light) is the major determinant of differences between stream types in algal standing crop, which in turn contributes to overall periphyton biomass differences. We found low levels of riparian canopy cover in constructed streams relative to reference streams because most constructed streams lacked woody plants in riparian areas (Table 1), a finding similar to that of Petty

et al. (2013) for streams constructed on mine sites in West Virginia. Only 2 constructed streams in our study (SCH and STO) were shaded by any riparian vegetation during the growing season, and only SCH was shaded by a dense overstory canopy of woody vegetation. In contrast, all reference stream riparian areas were characterized by mixed forests, with both eastern hemlock (*Tsuga canadensis*) and rhododendron (*Rhododendron maximum*) providing partial shade throughout the year. The greatest differences

Table 4. Spearman correlation coefficients  $|r| > 0.7$  for algal accrual rates ( $r_{\text{Chla}}/\text{d}$ ) and peak algal standing crop ( $\text{Chl } a_{\text{max}}$ ) vs environmental and benthic macroinvertebrate variables among constructed streams ( $n = 8$ ) for fall and summer short-term accrual periods. CV = coefficient of variation [ $\text{CV}\% = (\text{SD}/\text{mean}) \times 100$ ], from Kelvin-scale temperature.  $D_{50}$  is median streambed particle size. n/a = not applicable ( $|r| \leq 0.7$ ).

Structural Variable	Fall 14 Oct–16 Dec 2011		Summer 28 Jul–23 Aug 2012	
	$r_{\text{Chla}}/\text{d}$	$[\text{Chl } a]_{\text{max}}$ ( $\text{mg}/\text{m}^2$ )	$r_{\text{Chla}}/\text{d}$	$[\text{Chl } a]_{\text{max}}$ ( $\text{mg}/\text{m}^2$ )
Water chemistry ( $\text{mg L}^{-1}$ )				
[ $\text{HCO}_3^-$ ] dissolved	n/a	0.79	n/a	n/a
Water temperature ( $^{\circ}\text{C}$ )				
Median daily mean	n/a	0.71	n/a	n/a
Minimum daily mean	0.76	0.81	n/a	-0.79
CV daily mean (%)	-0.71	-0.81	n/a	n/a
Macroinvertebrates				
% Chironomidae	0.79	0.79	n/a	0.76
% 2-most-dominant taxa	n/a	n/a	0.81	n/a
% Predator	n/a	n/a	-0.88	n/a
Habitat and physical				
Bank stability	n/a	n/a	-0.79	n/a
Stream slope (%)	n/a	n/a	0.88	n/a
Baseflow wetted depth (cm)	-0.71	-0.76	n/a	n/a
Bankfull depth (cm)	-0.91	-0.81	n/a	-0.90
$D_{50}$ (mm)	0.84	n/a	n/a	n/a
In-line valley fill cover (%)	n/a	n/a	0.71	n/a

in algal standing crop and periphyton biomass between stream types occurred during summer, when stream-type disparities in riparian cover were highest. The partially shaded constructed streams (SCH and STO) had algal accrual rates most similar to reference streams (Table S2), which further suggests that periphyton differences were a result of shade. Additionally, in reference streams the mean algal standing crop from long-term studies nominally exceeded that of constructed streams on only 2 occasions (March and April 2012). Peak algal biomass and gross primary production generally occur in March and April in Appalachian headwater streams (Roberts et al. 2007), and these peaks were likely augmented by blow-downs of large coniferous trees (*T. canadensis*) at 2 reference reaches during the 2<sup>nd</sup> year of this study. These canopy openings may have been similar to those caused by logging, which has been shown to cause elevated periphyton biomass and algal standing crop (Hansmann and Phinney 1973, Lyford and Gregory 1975). Together, these lines of evidence suggest that light differences between stream types is a primary driver of differences in the accrual of algal standing crop and periphyton biomass in our study.

Contrary to our expectations, however, overstory canopy cover was not strongly associated with peak algal stand-

ing crop and accrual in constructed streams (Table 4). Dense herbaceous vegetation along stream margins, steep banks of deep stream channels, and the occasional presence of aquatic vegetation may have partially shaded the benthic environment in some of the narrow, constructed streams in our study (e.g., STO). Other authors have documented that stream-surface PAR is not consistently correlated with canopy cover (DeNicola et al. 1992), and that variability in water-column light attenuation can be affected by fine suspended sediments (Davies-Colley et al. 1992), dissolved OM (Kirk 1983), and channel depth (Kirk 1983, Hill 1996). However, in our study, canopy-cover differences between constructed and reference stream categories were much greater than the small differences observed among constructed streams (7 of 8 had canopy cover <25%). We, therefore, do not interpret the lack of strong correlation between canopy cover and algal accrual or peak biomass among the constructed streams as invalidating our conclusion that light is the primary factor driving periphyton differences between reference and constructed stream categories. Rather, our results are consistent with findings of other authors (Kirk 1983, Davies-Colley et al. 1992, Hill 1996) and suggest that canopy cover was likely an inadequate surrogate for PAR.

Temperature seemed to be a secondary driver of greater periphyton and algal standing crops in constructed than in reference streams during the fall season. Constructed streams were warmer than reference streams in terms of both average and mean minimum daily temperatures, as is typical in areas subjected to mining in eastern Kentucky and southern West Virginia (Fritz et al. 2010, Petty et al. 2013). However, if temperature were the primary driver of periphyton differences throughout the year, increasing temperatures from spring through summer should have led to increasing long-term periphyton accrual for both stream types. Instead, both AFDM and Chl *a* increased in constructed streams from March through August, but remained relatively constant in reference streams over the same time period (Fig. 2). Moreover, minimum stream temperature and peak algal standing crop were associated negatively across constructed streams during summer (Table 4). Thus, increased temperature does not appear to be a major contributor to accrual or standing crop in constructed streams during summer. Conversely, several water-temperature variables were associated positively with benthic algal accrual rates and peak standing crops in constructed streams during the fall, and adjusting for temperature differences ( $r_{\text{Chla}}/\text{degree-day}$ ) minimized differences in algal accrual rate during the fall season (Fig. 3B, C). Thus, the constructed streams' elevated temperatures seemed to stimulate periphyton growth in the fall. This result is consistent with previous studies that found temperature can be of secondary importance, relative to light, as a limiting factor (Phinney and McIntire 1965, McIntire 1968, Hornick et al. 1981, Bothwell 1988). Furthermore, this result corroborates previous research that shows controls on periphyton productivity vary seasonally (Rosemond 1993, Rosemond et al. 2000).

Constructed streams also had higher  $\text{NO}_3 + \text{NO}_2\text{-N}$  concentrations than reference streams (Table 1), similar to findings by Petty et al. (2013); but we do not think  $\text{NO}_3 + \text{NO}_2\text{-N}$  concentrations are likely to be a primary driver of periphyton differences between stream types. Differences in  $\text{NO}_3 + \text{NO}_2\text{-N}$  concentration between stream types were smallest during summer months, when the difference in periphyton and algal biomass between stream types was highest. Furthermore,  $\text{NO}_3 + \text{NO}_2\text{-N}$  concentrations were not correlated with biomass or accrual within the population of constructed streams during the summer and fall seasons. Additionally, SRP was low for all samples in both stream types, suggesting that P was more limiting than N. Furthermore, several studies examining the influence of light and nutrient availability on periphyton in streams have shown that light is a primary bottom-up driver of standing crops (Lowe et al. 1986, Hill and Knight 1988, Mosisch et al. 2001, but see Mosisch et al. 1999).

Our results suggest that grazing pressure is lower in constructed streams, which could contribute to differences

in algal standing crop and periphyton biomass between stream types. Top-down effects of herbivory can be important determinants of periphyton biomass or algal standing crop in some streams (Winterbourn 1990, Rosemond 1993, Feminella and Hawkins 1995, Hill et al. 1995, Rosemond et al. 2000). Here, we found lower relative abundance of scraper-grazer macroinvertebrates in constructed streams than in reference streams (Table 1; Krenz et al. 2016). It is plausible that the reduced relative abundance of grazers in constructed streams (Table 1) released top-down grazing pressures, although we did not measure density of scraper-grazer macroinvertebrates. Previous studies have suggested that Ephemeroptera, which are prominent components of scraper-grazer FFGs within Appalachian headwaters, are less abundant in mining-influenced streams with elevated conductivity than in low-conductivity reference streams (Pond et al. 2008, 2014). The constructed streams in our study are higher in conductivity and have lower relative abundances of Ephemeroptera than our reference streams (Krenz et al. 2016). Previous work, however, found that grazers were more abundant in streams constructed on West Virginia mine sites than in reference streams (Petty et al. 2013).

In conclusion, our results show that there are differences in periphyton structure and function between recently constructed streams and reference streams. Furthermore, our findings imply that bottom-up factors are largely responsible for these differences. Light availability in constructed streams during leaf-on seasons is the primary driver of algal standing crop and differences in accrual, though elevated temperatures in reference streams also contribute in the fall. Woody riparian canopy cover can provide shade, reducing both benthic irradiance and thermal energy inputs from insolation. Therefore, practices that promote establishment of woody vegetation in the riparian areas of streams constructed as compensatory mitigation in the Appalachian coalfield may accelerate establishment of periphyton structural and functional attributes resembling those of forested headwater streams.

## ACKNOWLEDGEMENTS

Author contributions: RJK conceived and executed the study, including data collection, analysis, and initial manuscript preparation. SHS and CEZ directed and oversaw study design, data collection, and data analysis. CEZ, RJK, and SHS prepared the manuscript.

We thank Lance DeBord, Mark Sproles, Phil Mullins, Eddie Clapp, Dan Evans, Robert Northington, Terry Owens, Craig Kade-ravek, and Tony Timpano, who aided stream selection and access; Dave Mitchem, Bobbie Niederlehner, and Patricia Donovan for direction with lab and geospatial analyses; Jackson R. Webster and E. Fred Benfield for advice concerning deployment of artificial substrates; Kyle Dost and Lindsey Nolan for scraping thousands of tiles; and the Powell River Project for funding this work.

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