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## Acid mine drainage affects the development and function of epilithic biofilms in streams

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**Abstract.** Acid mine drainage (AMD) is a legacy of historical coal mining that affects several thousand stream kilometers around the world by contributing high loads of acidity,  $\text{SO}_4^{2-}$ , metals, and other cations. Most research has focused on how AMD affects diversity and structure of various biological assemblages, whereas considerably less has focused on functional processes in streams. We investigated how AMD from abandoned coal mines affects epilithic biofilm development and function. Algal biomass and accrual rates were significantly lower in AMD-affected stream reaches than in control streams. Biomass and accrual rates were lowest at intermediate AMD-affected sites (pH = 5.1–5.9), probably because copious amounts of  $\text{Al}(\text{OH})_3$  precipitates smothered the benthic habitat. Ratios of  $\beta$ -glucosidase (GLU): $\beta$ -xylosidase (XYLO) were significantly correlated with algal biomass ( $r = 0.60$ ,  $p < 0.01$ ), probably because algae are important sources of C that is readily broken down by bacterial GLU activity, whereas XYLO is mostly associated with C of allochthonous origin. Thus, reduced algal biomass could indirectly exacerbate AMD effects on bacterial function by providing less C needed for cellular functions. Ratios of phosphatase:leucine aminopeptidase significantly increased as pH decreased ( $R^2 = 0.88$ ,  $p < 0.01$ ). This increase indicated potential P limitation or stressful conditions for microbial communities because  $\text{PO}_4\text{-P}$  readily adsorbs to metal hydroxides, rendering it biologically inaccessible. Ratios in 3 of 4 control streams indicated that N limitation may be more typical in unaffected streams of the region. Low algal biomass and potential P limitation of biofilms may indicate that AMD-affected streams have reduced capabilities to retain nutrients and energy needed to support healthy ecosystems. Considering the links between biofilm structure and function can provide a framework for developing management strategies to restore and conserve ecosystem processes, such as nutrient retention and spiraling, energy flow, food webs, and biodiversity.

**Key words:** extracellular enzyme activity (EEA), nutrient limitation, phosphatase, phosphorus, nitrogen, carbon, ecosystem function, succession, algae, periphyton, acid mine drainage (AMD), stream.

Human activities, such as resource extraction, agriculture, and urbanization, negatively affect the structure and functions of lotic ecosystems by altering their physical and chemical characteristics (Allan 2004, Sweeney et al. 2004). Epilithic habitats are especially important to ecosystem processes because they are often the most abundant biofilm-supporting substrata in wadeable streams (Lock et al. 1984). Characterizing the structure and functions of biofilms contributes to a better understanding of how human activities affect ecosystem processes, such as nutrient cycling (Davis and Minshall 1999, Hoellein et al. 2007) and energy flow (Hall et al. 2000, Augspurger et al. 2008), which

combine to affect the presence and abundance of other aquatic organisms (Fisher and Likens 1973, Power et al. 1985, Death and Zimmerman 2005).

Acid mine drainage (AMD) is a legacy of pre-regulation coal mining that impairs several thousand stream kilometers in many Appalachian states, such as Ohio, West Virginia, and Pennsylvania (Herlihy et al. 1990, USEPA 2000), and in other parts of the world (Bray et al. 2008). Pyritic materials ( $\text{FeS}$ ) in coal waste and overburden exposed to  $\text{O}_2$ , water, and microbial processes form sulfuric acid, which leads to extremely low pH that causes many metals in coal deposits and geologic formations, especially  $\text{Fe}^{2+}$  and  $\text{Al}^{3+}$ , to move into solution and flow through surface and subsurface waters (Mills 1985, Carroll et al. 2003). As pH increases with inputs from downstream alkaline waters, dissolved  $\text{Fe}^{2+}$  and  $\text{Al}^{3+}$  precipitate as hydroxide complexes that smother benthic substrata of streams (Younger et al. 2002).

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Effects of AMD from abandoned coal mines on the structure and diversity of stream organisms, such as fish, macroinvertebrates, and diatoms, have been studied extensively (Letterman and Mitsch 1978, DeNicola and Stapleton 2002, Petty et al. 2010, Smucker and Vis 2011), but considerably less research has focused on functional aspects, especially of epilithic biofilms. Most research on functional processes has been focused on streams affected by acidification from noncoal-mining sources and has emphasized leaf-litter breakdown (Niyogi et al. 2001, Carlisle and Clements 2005, Simon et al. 2009). The chemical and physical stressors caused by AMD from abandoned coal mines can affect primary production (Verb and Vis 2000, Simmons et al. 2005) and probably influence other functional processes, such as organic matter processing and nutrient uptake, as seen in streams affected by other sources of acidity (Niyogi et al. 1999, 2002, 2003, Simon et al. 2009).

Metal precipitates can directly reduce algal biomass by their physical effect on benthic habitats (Niyogi et al. 1999). Precipitates also reduce bioavailability of  $\text{PO}_4\text{-P}$ , which adsorbs to metal hydroxides (Moore and Miller 1994, Kopáček et al. 2000, Simmons 2010), and can cause further stress to benthic microbial communities. Reduced algal biomass could exacerbate AMD effects on bacterial function because algae are important sources of particulate and dissolved organic C (Kaplan and Bott 1982, Wyatt et al. 2010) through photosynthetic activity, exudates, senescence, and lysed cells (Murray et al. 1986, Espeland et al. 2001).

Algal biomass and extracellular enzyme activities (EEAs) can provide important structural and functional information on anthropogenic effects on biofilms (Stevenson et al. 2008, Scott et al. 2009b) because of tight algal-bacterial coupling in biofilms (Haack and McFeters 1982, Murray et al. 1986, Scott et al. 2009a). Most organic matter, polymers, and proteinaceous compounds require extracellular enzyme hydrolysis because they are too large for transport across microbial cell membranes (Chróst 1991). EEAs provide a direct measure of the physiological needs of microbial organisms, primarily bacteria, in epilithic biofilms for C and nutrients that are essential for cellular activity and growth when readily available inorganic forms are in short supply (Sinsabaugh and Foreman 2001, Sterner and Elser 2002).

We studied algal biomass accrual and extracellular enzyme activities to document how AMD affects the structural and functional formation of epilithic biofilms and nutrient and C dynamics during development. Characterizing succession provides information on biofilm recovery after disturbances, such as high-

flow events that are common in streams, and on how biofilms attain their mature state when they are most often sampled for ecological or biomonitoring studies. Our objectives were to document and describe development and function of stream biofilms along a gradient of AMD pollution. Our hypotheses were that: 1) rates of algal biomass accrual and biomass of mature biofilms would be lower in AMD-affected than in unaffected streams because of chemical and physical changes associated with AMD, 2) increased algal biomass would be accompanied by shifts in C-acquiring enzymes because of changing availability of autochthonous C, and 3) as AMD effects became more severe, ratios of P- to N-acquiring EEAs would increase because of greater P limitation resulting from  $\text{PO}_4\text{-P}$  adsorption to metal hydroxide precipitates and biological unavailability. We also investigated the effects of metal precipitation on the recovery of biofilms in a 4.8-km reach.

## Methods

### *Study sites*

During September 2008, we selected 7 sites in 5 streams in the Western Allegheny Plateau region of southeastern Ohio based on physical habitat (consistent riffle depth, rocky substrata, current velocity, and open canopy) and chemistry (representing an AMD-effect gradient as indicated from historical monitoring and use of pH as a proxy of AMD severity). To ease communication of results, sites were given abbreviated names representing effect type and severity (Table 1). Carbondale Creek (site A1) near Carbondale had pH  $\sim 3.2$  and observable  $\text{FeOH}_3$  precipitates, East Branch (site A2) near Coonville had pH  $\sim 4.9$  with no observable metal hydroxide precipitates during the study, and Spencer Hollow near Murray City was sampled immediately downstream of an abandoned mine discharge where substantial  $\text{AlOH}_3$  precipitation was occurring. We selected this stream to study the downstream recovery of biofilms from Al precipitates, and we sampled 3 sites that were 1.6 km (site D1), 3.2 km (site D2), and 4.8 km (site D3) downstream of the AMD discharge with pH 5.9, 5.4, and 5.1, respectively. Little Monday Creek (site A3) north of Greendale represented a natural downstream dilution of AMD effects and had pH 7.5 but still had elevated conductivity and  $\text{SO}_4^{2-}$ . Clear Creek (site C1) west of Logan had pH 8.4 and was a control stream with no coal mining effects in the upstream watershed. All streams were within a 15-km radius of each other and had water temperatures between 18–20°C. We also used data from 3 streams with pH 7.8 to 8.1 from a previous study done in August 2008 in which

TABLE 1. Mean ( $\pm 1$  SE) values for chemistry variables ( $n = 6$  [day of tile deployment and days 5, 12, 19, 26, and 33] at each site). Site abbreviations: A1 = Carbondale Creek; A2 = East Branch; A3 = Little Monday Creek; D1, D2, D3 = Spencer Hollow 1.6 km, 3.2 km, and 4.8 km downstream of acid mine discharge, respectively; C1 = Clear Creek (control stream); EC = electrical conductivity.

Site	pH	EC ( $\mu\text{S}/\text{cm}^2$ )	$\text{SO}_4^{2-}$ (mg/L)	$\text{NO}_3\text{-N}$ (mg/L)	$\text{PO}_4\text{-P}$ (mg/L)	$\text{NO}_3\text{-N}:\text{PO}_4\text{-P}$
A1	$3.15 \pm 0.02$	$1330 \pm 68$	$573 \pm 114$	$0.04 \pm 0.01$	$0.29 \pm 0.07$	$0.16 \pm 0.05$
A2	$4.85 \pm 0.02$	$1565 \pm 54$	$827 \pm 163$	$0.11 \pm 0.04$	$0.21 \pm 0.05$	$0.59 \pm 0.13$
A3	$7.53 \pm 0.04$	$1100 \pm 60$	$135 \pm 24$	$0.03 \pm 0.004$	$0.07 \pm 0.02$	$0.99 \pm 0.44$
D1	$5.85 \pm 0.09$	$903 \pm 26$	$302 \pm 49$	$0.06 \pm 0.03$	$0.09 \pm 0.04$	$1.20 \pm 0.35$
D2	$5.44 \pm 0.12$	$872 \pm 15$	$257 \pm 50$	$0.11 \pm 0.03$	$0.12 \pm 0.04$	$2.04 \pm 0.72$
D3	$5.13 \pm 0.06$	$880 \pm 14$	$272 \pm 60$	$0.07 \pm 0.01$	$0.09 \pm 0.02$	$1.39 \pm 0.66$
C1	$8.41 \pm 0.06$	$392 \pm 7.9$	$17 \pm 1.0$	$0.35 \pm 0.05$	$0.14 \pm 0.02$	$2.82 \pm 0.45$

we sampled mature biofilms grown on the same type of tile deployed for a similar length of time as the present study (Smucker et al. 2009). We used these streams as additional controls to improve inferences of the structure and function of minimally affected, nonAMD streams in the region.

At each site, we deployed  $5.08 \times 5.08$  cm ( $25.8$  cm<sup>2</sup>) unglazed ceramic tiles in a single riffle with 48 tiles in the upstream  $\frac{1}{2}$  and 48 in the downstream  $\frac{1}{2}$  of the riffle. Ceramic tiles provide a uniform substratum among streams, support biofilms with high similarity to those on natural substrata, and have low variability relative to natural substrata (Lamberti and Resh 1985). For each group of tiles, we attached 4 strips of tiles ( $2 \times 6$  tile arrangement, separated by 8 cm from adjacent strips) to wire fencing and staked them flush with the stream bottom. Grazers can have variable effects on algal biomass (Steinman and McIntire 1990, Steinman 1996, Wellnitz and Rader 2003), but we did not exclude them because of the difficulty associated with setting mesh that would exclude macroinvertebrates without altering flow or becoming clogged by leaves and debris. These obstacles are more readily circumvented in artificial stream mesocosms. However, we did not observe active grazing or evidence thereof on any tiles, and grazers probably had minimal effects on algal biomass accrual when compared to the effects of AMD, although AMD could have affected algal biomass indirectly by reducing macroinvertebrate diversity and abundance (Niyogi et al. 2002). Macroinvertebrates were rarely observed on tiles, and only *Chironomus* species in the worst AMD site (A1) had to be removed from tiles prior to analysis.

We collected 3 replicates, each consisting of 2 upstream and 2 downstream randomly selected tiles, 5, 12, 19, 26, and 33 d after deployment. Mature epilithic biofilms typically develop well before 28 d (Gale et al. 1979). We included tiles from upstream and downstream in each replicate to provide greater representation of riffle conditions and to reduce the influence of small-scale variability. When sampling, we

removed tiles carefully from the wire fencing, placed them in containers, and sealed the containers with a lid before removing them from the stream. Keeping the tiles in watertight, sealed containers reduced disruption to biofilms during transport to the laboratory. We transported tiles on ice for analyses within 24 h.

At the time of each site visit, we measured conductivity, pH, and temperature in situ with handheld probes (Waterproof ECTestr<sup>®</sup> and pHTestr 30<sup>®</sup>; Oakton, Vernon Hills, Illinois). We also collected filtered samples ( $0.45\text{-}\mu\text{m}$  pores; Millipore<sup>®</sup>, Billerica, Massachusetts) for chemical analyses of the water column at the upstream end of riffles and immediately placed them on ice until returning to the laboratory for analysis. We measured  $\text{PO}_4\text{-P}$ ,  $\text{NO}_3\text{-N}$ , and  $\text{SO}_4^{2-}$  with US Environmental Protection Agency-approved protocols (APHA 1995).

#### EEAs

Enzymes of frequent interest in lotic systems include: phosphatase (PHOS), which hydrolyzes phosphoric monoesters to mineralize organically bound P into  $\text{PO}_4$ ; leucine aminopeptidase (LAMP), which hydrolyzes proteins and other amino acids to acquire N;  $\beta$ -glucosidase (GLU), which contributes to the hydrolysis of 1,4-linked  $\beta$ -D-glucose from  $\beta$ -D-glucoside residues and cellobiose; and  $\beta$ -xylosidase (XYLO), which degrades  $\beta$ -1,4-linked polymers of xylopyranose largely associated with plant cell walls and cellulose (Weintraub et al. 2007). PHOS:LAMP ratios are expected to shift in response to metabolic limitation by the availability of inorganic P or N (Sala et al. 2001, Hill et al. 2010b). The ratios of C-acquiring enzymes, primarily GLU and XYLO, are important for characterizing organic C processing in biofilms. Larger ratios typically indicate greater inputs of autochthonous C from algal photosynthate, cellobiose and exudates, and smaller ratios indicate a greater role of allochthonous sources of terrestrial origin in C dynamics (Romani and Sabater 2000).

We assayed EEAs within 24 h because activities can change significantly with longer storage duration (Smucker et al. 2009). We prepared samples for EEA measurements by removing the biofilm of each replicate group of 4 tiles with a razor blade and clean toothbrush. We diluted samples to 120 mL with filtered (0.45- $\mu$ m pore-size filters) stream water from the appropriate sample site. We homogenized each replicate for 30 s with a Tissue Tearor at high speed (BioSpec Products Inc., Bartlesville, Oklahoma), which dispersed cells without damaging membranes. We quantified EEAs in 96-well black polystyrene microplates with 300- $\mu$ L wells (12 columns  $\times$  8 rows; Whatman Inc., Florham Park, New Jersey) with analytical replicates for determination of mean fluorescence.

We used fluorescent methylumbelliferone (MUB)-linked substrates from Sigma-Aldrich (St Louis, Missouri) to measure PHOS (M8883), GLU (M3633), and XYLO (M7008) activities of biofilms and the fluorogenic substrate leucine 7-amido-4-methylcoumarin (L2145) to measure LAMP activity (Sigma-Aldrich). We measured fluorescence with a microplate fluorometer with excitation wavelength of 365 nm and emission wavelength of 455 nm (Synergy HT; BioTek, Winooski, Vermont). Preliminary experiments with EEAs from extra tiles from the sites and different substrate concentrations (10, 50, 100, 300, 600  $\mu$ M) over time indicated that 300  $\mu$ M (final concentration) with 20- to 40-min incubations at 20°C were appropriate for assays of these 4 enzymes. We made fresh substrate and standard solutions on the day of analysis because some substrates and fluorescent standards deteriorate within 3 d (DeForest 2009). We used MUB reference standards to convert fluorescence to EEA values, and we used negative controls of each substrate and stream water to account for fluorescence from nonbiofilm sources. We used quenches, which were biofilms mixed with MUB reference standards, to correct for the amount of fluorescence masked by samples. We determined EEAs as described in Smucker et al. (2009).

After homogenizing samples for enzyme assays, we filtered 1-mL aliquots onto Whatman glass-fiber filters (GF/F; 0.45- $\mu$ m pore size) and froze the filters until analysis for chlorophyll *a* as a measure of algal biomass. We immersed filters in 8 mL of 90% acetone for 18 h in the dark at 4°C (Arar and Collins 1997). We measured the fluorescence of the leachate with a TD-700 fluorometer (Turner Designs, Sunnyvale, California) to determine chlorophyll *a* concentration.

#### Data analysis

Chlorophyll *a*, enzyme activities, and ratios were  $\log_{10}(x + 1)$ -transformed to meet assumptions of

normality and homogeneity of variance (modified Levene equal-variance test) for parametric statistical tests. We used pH as proxy in analyses to represent the gradient of AMD effects. Chlorophyll *a* accrual data were nonlinearly related to time. Therefore, we used nonlinear regression of chlorophyll *a* vs time to test if accrual patterns differed as a result of AMD severity. We defined significant differences as nonoverlapping 95% confidence intervals among sites. We also used nonlinear regression to examine the relationship of mature biofilm chlorophyll *a* and pH. We conducted Pearson correlations between GLU:XYLO ratios and chlorophyll *a* to investigate whether increased algal biomass was related to the activities of C-acquiring enzymes. We used ternary plots to show the allocation of N-, P-, and C-acquiring enzyme activities among sites during early succession (days 5–12) and mature states of late succession (days 19–33). We conducted linear regression to characterize the relationship of PHOS:LAMP ratios and pH at study sites for the purpose of identifying whether P limitation was indicated by EEAs in AMD-affected streams. When discussing potentially P- or N-limited conditions inferred from biofilm EEAs, we used deviations from the well-established 1:7 (>1:4 P-limited or <1:10 N-limited thresholds) ratio of phosphatase to peptidase (Hill et al. 2006, 2010b).

## Results

Conductivity, pH, and concentrations of  $\text{NO}_3\text{-N}$ ,  $\text{PO}_4\text{-P}$ , and  $\text{SO}_4^{2-}$  were consistent within sites throughout the study (Table 1). Conductivity and  $\text{SO}_4^{2-}$  had greater variation than other chemistry variables in AMD-affected streams during the 33-d study, but the variation was much less than the differences among sites. In general, AMD sites had conductivities  $>2$  to  $4\times$  and  $\text{SO}_4^{2-}$  concentrations  $>8$  to  $48\times$  those of the control stream.  $\text{NO}_3\text{-N}:\text{PO}_4\text{-P}$  ratios were extremely low at all sites throughout the study, and ratios rarely exceeded 3:1 (Table 1), a result that indicated potential N limitation based on water chemistry (Davis and Minshall 1999).

Algal biomass accrual, as indicated by chlorophyll *a*, varied greatly among sites. Accrual rates were significantly lower in the AMD-affected streams than in the control stream, as indicated by nonoverlapping 95% confidence intervals (Fig. 1A). Sites downstream of the AMD discharge (D1, D2, D3) had the slowest rates of algal biomass accrual, and sites A2 and A3 were intermediate (Fig. 1B). Chlorophyll *a* of mature biofilms had a U-shaped relationship with pH. Chlorophyll *a* was greatest at sites C1, A1, and the 3 control streams from Smucker et al. (2009), intermediate at A2

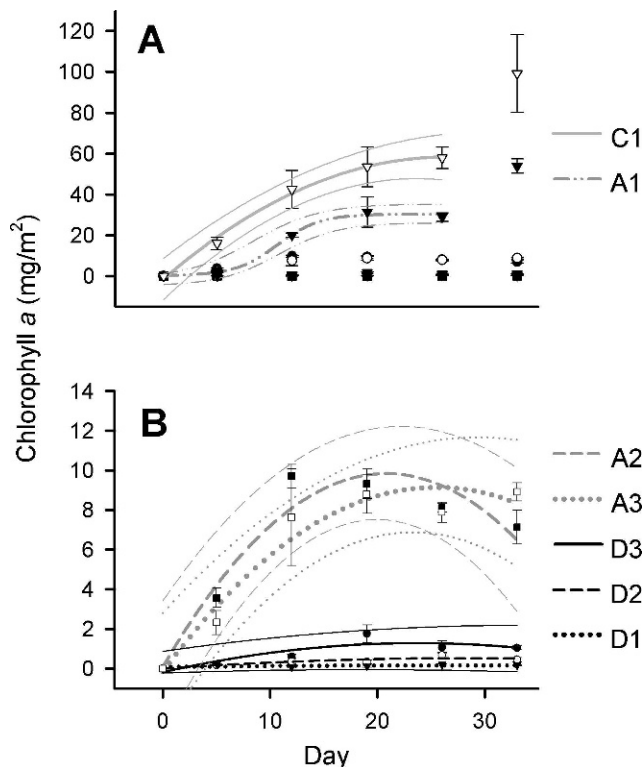


FIG. 1. Nonlinear regressions (95% confidence intervals) comparing algal biomass (chlorophyll *a*) accrual on ceramic tiles among sites. Data points are means ( $\pm 1$  SE,  $n = 3$ ) for each site on each sampling date. A.—Rates of accrual at sites C1 and A1. Day 33 was excluded from regressions on A1 and C1 because noticeably greater flow resulting from a rain event occurred on and after day 26 and potentially stimulated algal growth that was not observed at other sites (McIntire 1966, Horner and Welch 1981). B.—Magnification of sites A2, A3, D1, D2, and D3. For clarity and because 95% confidence intervals overlapped at sites D1, D2, and D3, only the 95% CI of D3 and 5% CI of D1 are shown.

and A3, and lowest at D1, D2, and D3 (Fig. 2). Based on data from all sites on all sampling days, ratios of GLU:XYLO increased significantly as chlorophyll *a* increased ( $r = 0.60$ ,  $p < 0.001$ ; Fig. 3).

Allocations of EEAs devoted to acquiring P, N, and C varied greatly among the 7 sites, and these allocations of EEAs changed most from early-successional to late-successional communities at AMD-affected sites (Fig. 4A, B). During early and late succession, sites C1 (means: 81 and 74%) and A3 (means: 58 and 60%) had the greatest percentage of total EEAs allocated toward N-acquisition. Sites D1, D2, and D3 were much more variable during early succession, but most EEAs were allocated toward C- (means: 26–65%) and P-acquisition (means: 10–51%). Site A2 varied between P- (mean: 42%) and N-acquisition (mean:

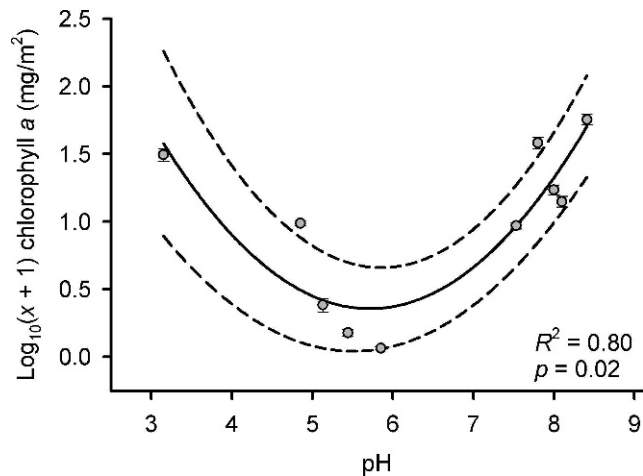


FIG. 2. Nonlinear regression (95% confidence intervals) showing the relationship between pH and  $\log_{10}(x + 1)$ -transformed means ( $\pm 1$  SE,  $n = 9$ ) of algal biomass (chlorophyll *a*) for days 19 to 26. Means of days 19 to 26 were used because they represented mature biofilms (see Fig. 1).

38%) during early succession, and EEAs at site A1 were mostly allocated toward P-acquisition (mean: 72%). The allocation of EEAs at all sites became much less variable during late succession (Fig. 4B). At sites C1, A3, and the 3 control streams from Smucker et al. (2009), EEAs were mostly devoted to N-acquisition (means: 60–93%) and some C-acquisition (3–30%). The 5 AMD-affected sites with pH lower than 5.9 had

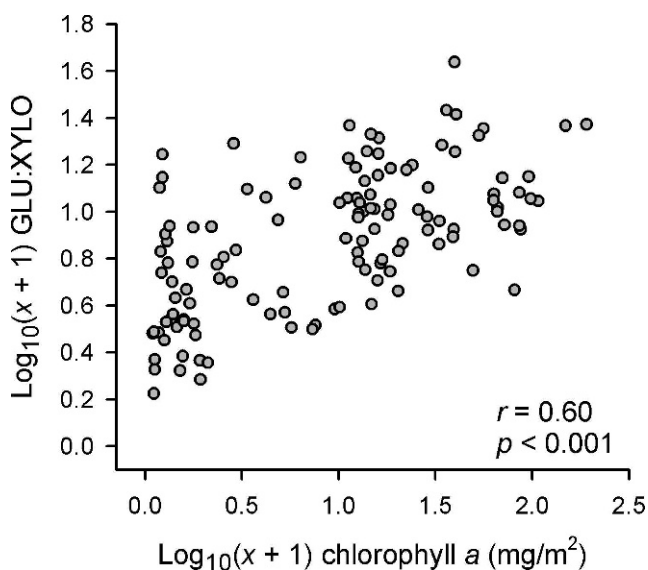


FIG. 3. Scatterplot showing the relationship of the  $\beta$ -glucosidase (GLU): $\beta$ -xylosidase (XYLO) ratios with algal biomass (chlorophyll *a*) for all samples ( $n = 125$ ).

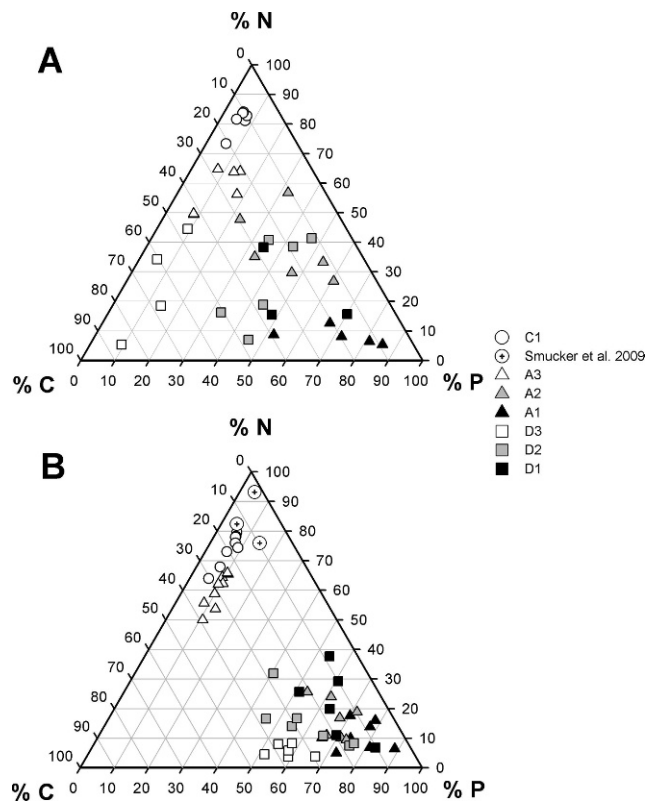


FIG. 4. Ternary plots showing % total measured extracellular enzyme activities allocated to acquiring P, N, and C at sites during early (days 5–12) (A) and late (days 19–33) (B) succession. Enlarged open circles with crosshairs = 3 control sites from Smucker et al. (2009).

most of their total EEAs allocated toward P-acquisition (means: 58–78%).

The PHOS:LAMP ratios at sites A1, A2, D1, D2, and D3 were >2:1. This ratio far exceeded 1:4 (ratios > 0.25), indicating P-limited conditions. The ratios of PHOS:LAMP at site A3 ( $0.17 \pm 0.012$ ) and at 1 of the 3 control streams in Smucker et al. (2009) with pH = 8.0 ( $0.21 \pm 0.03$ ) indicated neither P- nor N-limited conditions ( $0.1 < x < 0.25$ ), whereas the ratios at C1 ( $0.08 \pm 0.004$ ) and the other 2 control streams in Smucker et al. (2009) with pH 8.1 ( $0.05 \pm 0.004$ ) and 7.8 ( $0.06 \pm 0.004$ ) indicated potential N limitation (ratios < 0.10). The ratios of PHOS:LAMP significantly increased with decreasing pH ( $R^2 = 0.88$ ,  $p < 0.001$ ; Fig. 5). For all sites, the activity ratios of C:P- and C:N-acquiring enzymes were <3:1 and <3.3:1, respectively, with the exception of site D3, which had a C:N acquisition ratio = 7.2:1. For perspective, the expected ratio of C:P-acquiring enzyme activities is 60:1 and for C:N-acquiring enzyme activities 8:1, with values far exceeding these indicating potential C limitation (Hill et al. 2010a).

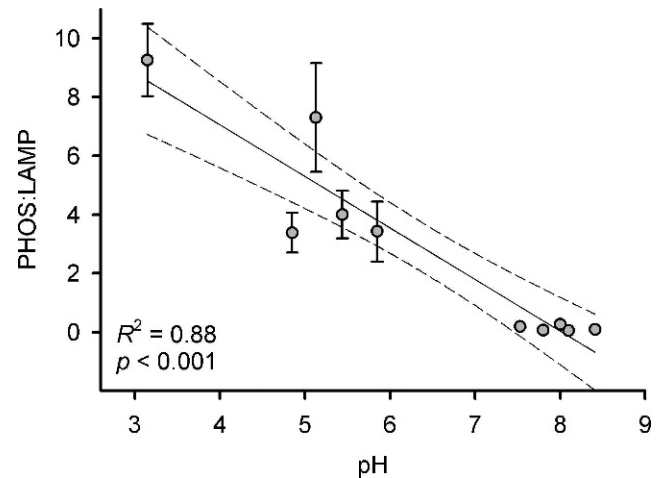


FIG. 5. Regression (95% confidence intervals) showing the relationship of mean ( $\pm 1$  SE) phosphatase (PHOS):leucine aminopeptidase (LAMP) ratios with stream pH.

## Discussion

### Algal biomass

Our hypothesis that AMD would reduce the rate of algal biomass accrual during succession and the biomass of mature biofilms was supported by our results. Algal biomass and accrual rates were significantly lower in all AMD-affected streams than in the control stream, but the response was more complex than a simple reduction of biomass and accrual with increasing severity of AMD pollution, probably because of the different natures of the chemical and physical stressors. A positive correlation between algal biomass and acidity has been observed in some studies (Muller 1980, Mulholland et al. 1986), but a decrease in biomass with increased acidity also has been observed (Kinross et al. 1993), as have conflicting relationships within a study (Verb and Vis 2000). A U-shaped relationship between pH and algal biomass of mature biofilms probably was observed in our study because of the severe physical effects of intermediate pH levels at which  $\text{Al}(\text{OH})_3$  precipitated from solution and covered the substratum.

Algal biomass, as measured by chlorophyll *a*, was almost nonexistent at sites with copious  $\text{Al}$  precipitates downstream of the AMD discharge (D1, D2, D3). Niyogi et al. (1999, 2002) found similar results in streams affected by metal mining with high levels of  $\text{Al}(\text{OH})_3$  precipitation. Beyond potential toxicity of dissolved metals to algae, the precipitates probably affected algal biomass accrual in 3 ways: 1) continuous precipitation in this stream probably reduced initial attachment and colonization by algae, which would then have altered future growth rates, 2) algal

cells that succeeded in colonizing faced the perpetual challenge of avoiding the continuous  $\text{Al}(\text{OH})_3$  deposition, and 3)  $\text{PO}_4\text{-P}$  probably adsorbed to precipitates, leading to P limitation, which would further slow algal growth rates and reduce biomass of mature biofilms. Algal biomass increased slightly farther downstream from the AMD discharge, probably because of less metal precipitation, but this increase was mostly negligible and biofilms were still far from recovered when compared to the control stream. Chemical stressors probably were associated with the decreased algal biomass and accrual rates in the other AMD streams.

### *C and nutrient dynamics*

Our hypothesis that increased algal biomass would be accompanied by shifts in different C-acquiring enzymes was well supported by the strong positive correlation between  $\text{GLU:XYLO}$  ratios and algal biomass. This correlation reinforced the hypothesis that algal exudates were important sources of C for bacteria. Low ratios indicate greater importance of allochthonous C sources requiring hydrolysis of hemicellulose by XYLO, whereas high ratios indicate greater importance of glucose obtained by hydrolysis of cellobiose, which originates mostly from algae (Romani and Sabater 2000). In addition to being a source of C, algal photosynthesis may increase the activities of extracellular enzymes by enhancing redox and pH conditions that could stimulate heterotrophic activity (Espeland et al. 2001, Rier et al. 2007). As a result, reduced algal biomass could indirectly exacerbate AMD effects on bacterial function by providing less C needed for cellular functions.

EEA allocations were much more variable within AMD-affected sites during early succession than during late succession or when compared to EEA allocations in the control stream and site A3 (pH = 7.5), which were more consistent during both time periods. This variability in AMD-affected streams may present potential problems for investigators conducting ecological studies and assessments or when creating management plans. If biofilms are sampled before they attain mature states, EEAs might represent conditions associated with succession rather than conditions associated with mature functioning states (>19 d) of biofilms. The differences in EEA allocation between early and late succession may indicate shifts in microbial community structure or resource availability. In mature biofilms, we found that substantial differences existed among sites in allocations of EEAs to resource acquisition. In control streams and site A3 (pH 7.5), most of the total EEA

allocation was toward N-acquisition, whereas in AMD-affected streams, most of the total EEA allocation was toward P-acquisition.

PHOS:LAMP ratios provide excellent evidence for nutrient limitation and stress in microbial communities (Sala et al. 2001, Hill et al. 2006). Our hypothesis that microbial biofilms in AMD-affected streams are stressed by P limitation was strongly supported by elevated PHOS:LAMP ratios in these streams. If a readily available inorganic nutrient were in surplus, organisms would not expend energy to release extracellular enzymes to hydrolyze compounds for acquiring that nutrient. For example, PHOS activity often is low when excess P is present (Klotz 1992, Scott et al. 2009b), and PHOS activity increases with declining P availability (Wright and Reddy 2001, Stevenson et al. 2008). Streams with PHOS:LAMP ratios > 0.25 (1:4) show strong indication of P limitation (Hill et al. 2006, 2010a, b), and the 5 AMD-affected sites with pH  $\leq$  5.9 had ratios much larger than this (all > 2:1). P limitation probably was caused by adsorption of  $\text{PO}_4\text{-P}$  to metal hydroxides, which subsequently rendered it biologically inaccessible (Moore and Miller 1994, Tanada et al. 2003, Simmons 2010). The significant negative relationship between PHOS:LAMP ratios and pH supports this finding because higher concentrations of metals often are present in more severely affected AMD streams with lower pH. Elevated PHOS activity has been observed in other acidic rivers with high concentrations of Al (Gross 2000, Sabater et al. 2003), and elevated microbial PHOS activity was associated with increasing acidity in a recent study on leaf breakdown along an acidity gradient in Appalachian streams (Simon et al. 2009).

We observed a disconnect between the concentrations of  $\text{PO}_4\text{-P}$  in the water column and what was biologically available in the biofilms.  $\text{PO}_4\text{-P}$  concentrations in AMD-affected sites were similar to or higher than (e.g., sites A1 and A2) in control streams, but PHOS:LAMP ratios in biofilms were several times greater in AMD-affected streams than in control streams. In addition,  $\text{NO}_3\text{-N:PO}_4\text{-P}$  ratios in the water column indicated that N limitation was more likely than P limitation. This mismatch probably occurred because P was bound to metal hydroxides by the time it reached the benthos or because the coating of metal hydroxide precipitates prevented biologically available  $\text{PO}_4\text{-P}$  from diffusing into the biofilm matrix. As a result, the unavailability of inorganic P probably led to an increase in PHOS activity as organisms attempted to access organically bound P. Our result shows the utility of quantifying EEAs as a means to gain insight into stream nutrient dynamics that might



be missed through use of other measures or water chemistry alone (Hill et al. 2010a, b). A similar disconnect between nutrient ratios in the water and sediment enzyme activities was found in large rivers (Hill et al. 2010a).

LAMP activity increases with declining availability of inorganic N (Montuelle and Volat 1998, Ainsworth and Goulder 2000). PHOS:LAMP ratios were <0.10 (1:10) in the control stream and 2 of the 3 previously studied streams, a result that indicated potential N limitation (Hill et al. 2006). This conclusion is supported by the extremely low  $\text{NO}_3\text{-N}:\text{PO}_4\text{-P}$  ratios in the water column at all sites. N limitation could be the typical condition in the Western Allegheny Plateau, but further research on more sites throughout the region and nutrient addition/uptake experiments are needed to verify this possibility. Streams in other regions of Appalachia tend to be N limited (Hill et al. 2010b).

#### *Potential implications of AMD effects*

Our results showed that the physical and chemical effects of AMD were strongly associated with major shifts in the structure and function of benthic biofilms. Algal biomass was lower and nutrient and C-acquiring enzyme activities were different in AMD-affected streams than in least-impaired AMD and control streams. Algal biomass reduction associated with AMD has implications for structural-functional links in biofilms that could affect ecosystem processes, such as nutrient retention and spiraling, flow of energy, and food webs. Healthy communities are crucial to retention, processing, and transport of nutrients and organic matter (Newbold et al. 1982, Peterson et al. 2001). Impairment of these functions can negatively affect downstream ecosystems and have consequences for society (Meyer 1997). The potentially severe P limitation and reduced biomass of biofilms in AMD-affected streams probably contribute to inefficient nutrient uptake and longer nutrient spirals, a pattern observed in streams affected by other anthropogenic stressors (Martí et al. 2004, Grimm et al. 2005, Newbold et al. 2006). Reduced ability of upstream biofilms to retain nutrients could compound the downstream effects of nutrient loading. Biotic uptake and retention of nutrients are important functions of aquatic ecosystems, and enhancing them can be a goal of stream restoration (Roberts et al. 2007). We did not study nutrient spiraling or uptake rates. However, low algal biomass and severe P limitation (shown by PHOS:LAMP ratios) provided strong evidence for the reduced capabilities of AMD-affected streams to

retain the nutrients and energy needed to support healthy ecosystems. AMD stressors also have obvious and direct effects on the presence, abundance, and biomass of macroinvertebrates and fish regardless of biofilm processes (e.g., fish can be absent because pH is too acidic and not necessarily because of food quantity and quality), and these effects can indirectly affect algal biomass (Niyogi et al. 2002).

AMD is a serious environmental problem that impairs >7200 km of streams in Appalachia (USEPA 2000), which have been viewed as limited-resource waters with no immediate prospect for reclamation. However, remediation measures, such as CaO dosers, alkalinity-generating slag beds, and retention ponds or wetlands that allow settling of metals before water enters streams, are promising approaches to reducing the effects of AMD (Hedin et al. 1994, Skousen et al. 1998, Simmons et al. 2002). Removing or reducing metal precipitates whenever possible before treated water is released would decrease the extent of downstream effects on biofilms. Management strategies for restoring and conserving ecosystem processes in streams should take into account the links between biofilm structure and function.

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