2021 *NORTHEASTERN NATURALIST* 28(1):28–48

Predictors of Biofilm Biomass in Oligotrophic Headwater Streams

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Abstract - Biofilm forms the base of food webs as an autochthonous resource in nutrient-poor, heavily shaded headwater streams. However, we know little about the influence of bottom-up and top-down controls on the standing stock of biofilm biomass in headwater streams. We used spatially extensive stream surveys and pre-existing stream chemistry data to assess the influence of potential bottom-up and top-down controls of biofilm biomass in the oligotrophic headwater streams of the Hubbard Brook Experimental Forest (HBEF), NH. The potential bottom-up controls we considered were canopy cover and aspect (determinants of available radiation at the stream surface), available nutrients (e.g., nitrogen, phosphorus), physical habitat characteristics (e.g., stream width, substrate), and physico-chemical properties of water (e.g., water conductivity, pH). The potential top-down controls we examined were benthic macroinvertebrate biomass and stream salamander occurrence. Salamanders may affect biofilm biomass indirectly by preying on benthic macroinvertebrate consumers. We used stepwise multiple linear regression to assess the relative importance of these variables in predicting biofilm biomass and found that biofilm increased with light availability (as indicated by canopy cover and aspect), nutrient availability, and lower acidity, suggesting that the autotrophic components of biofilm may be particularly important. Our top-down control indices were unrelated to biofilm biomass; however, more intensive studies on top-down controls are needed in these systems.

Introduction

Biofilm, the microbial community covering stream substrates, is an integral part of stream ecosystems, forming the base of stream food webs and controlling nutrient cycling (Battin et al. 2003, 2016; Mulholland et al. 1994). Many taxa consume stream biofilms, making it an essential resource fueling secondary productivity (Feminella and Hawkins 1995, Guo et al. 2018, Merrit and Cummins 1996), even in forested headwater streams with high inputs of terrestrially derived organic matter (Hall and Meyer 1998, Hall et al. 2001, McNeely et al. 2006). Biofilms are also important sites of nutrient uptake and transfer into stream food webs, particularly for headwater streams, which are often the first sites of terrestrial nutrient inputs (Lowe and Likens 2005, Peterson et al. 2001).

Historically, studies of stream biofilms have focused on the autotrophic component of biofilm (i.e., green algae, diatoms, cyanobacteria; Battin et al. 2016), often using chlorophyll-*a* concentration as an index of abundance and biomass. However, the autotrophic and heterotrophic (i.e., bacteria, fungi, protozoans) components

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Manuscript Editor: Daniel Hewins

generally co-occur in a matrix of extracellular polymeric substances (EPS) that they produce (Flemming and Wingender 2010, Flemming et al. 2016, Hoagland et al. 1993). The resulting physical structure, with its increased sorption ability and retention of extracellular enzymes, can alter the availability of water, light, and oxygen as well as increase metabolic efficiency, nutrient uptake, and resistance to desiccation and high-flow events for its members (Flemming et al. 2016, Hall-Stoodley et al. 2004, Roche et al. 2017). The EPS matrix can make up the majority of biofilm mass and is consumed along with heterotrophic and autotrophic components when taxa feed on biofilms (Flemming and Wingender 2010, Hall and Meyer 1998, Lawrence et al. 2002). Given these integral roles in food webs and nutrient processing, our understanding of stream ecosystems will benefit from greater knowledge of what controls biofilm biomass, including all its subcomponents.

Our extensive understanding of stream autotrophs, and emerging understanding of the combined auto- and heterotrophic components of biofilm, suggest that there are several bottom-up factors that could influence stream biofilm biomass, including canopy cover and aspect (as determinants of available radiation at the stream surface), available nutrients (e.g., nitrogen, phosphorus), physical habitat characteristics (e.g., stream width, substrate, gradient), and physicochemical properties of water (e.g., water conductivity, pH). Light is important for stream autotrophs (Schiller et al. 2007, Ylla et al. 2009), which are also often limited, or co-limited, by nitrogen and phosphorus (reviewed in Francoeur 2001, Hillebrand 2002, Lamberti 1996). Calcium and magnesium are important for biofilm attachment, perhaps by strengthening the biofilm's EPS matrix, although their role in limiting biofilm production has been less studied than that of nitrogen and phosphorus (Flemming et al. 2016, Geesey et al. 2000, Song and Leff 2006). Dissolved inorganic carbon (DIC) can affect periphyton growth (Fairchild and Sherman 1993, Vinebrooke 1996), and silica is important for diatom production (Carrick and Lowe 2007, Grady et al. 2007, Hill and Webster 1982). The heterotrophic component of stream biofilms (i.e., heterotrophic bacteria, fungi, protozoans) use dissolved organic carbon (DOC) as a food source, obtained from stream water or from their autotrophic neighbors (Cole 1982, Romani and Sabater 1999, Romani et al. 2004). Additional categories of bottom-up variables important for stream biofilms are physical habitat (e.g., substrate, gradient, water-flow conditions; Cardinale et al. 2002, Singer et al. 2010) and stream chemistry (e.g., pH; Lear et al. 2009, Ledger and Hildrew 2001).

Top-down control of biofilm biomass can result from consumption of biofilm by omnivorous benthic macroinvertebrates that ingest bacterial, fungal, and EPS components in addition to the autotrophic component of biofilms (Cummins and Klug 1979, Feminella and Hawkins 1995, Hall and Meyer 1998). There is some evidence that in oligotrophic systems, where lower productivity supports lower numbers of primary consumers, the importance of these top-down controls may be reduced relative to bottom-up controls (Dufour and Torréton 1996, Pace et al. 1999, Thelaus et al. 2008). However, this is not always the case (Gasol et al. 2002), and assessments of bottom-up and top-down factors in lotic systems have generally focused on the autotrophic component of biofilm (reviewed in

Hillebrand 2002) —an emphasis that might tend to highlight the importance of bottom-up effects. Top-down controls can also be indirect, such as through the consumption of benthic macroinvertebrates by fish (Biggs et al. 2000, Lamberti 1996, Winkelmann et al. 2014).

Here we use a combination of stream surveys and pre-existing stream chemistry data to assess the influence of bottom-up and top-down controls of stream biofilm biomass at the Hubbard Brook Experimental Forest (HBEF) in central New Hampshire. The HBEF streams are nutrient poor and heavily shaded (Likens 2013), making biofilms a potentially important in situ resource (Mayer and Likens 1987). Biofilms in headwater streams at the HBEF are dominated by heterotrophic members, as reflected in production/respiration ratios (P:R ratios), cell counts, and contributions to benthic macroinvertebrate diets (Burton et al. 1988, Hall et al. 2001, Webster et al. 2003). Previous work at the HBEF has focused on bottom-up controls of the autotrophic component of biofilm, and has usually taken place in only 1 or 2 focal streams. These studies have found conflicting effects by both nutrients (i.e., nitrogen, phosphorus; Bernhardt and Likens 2004, Chadwick and Huryn 2005, Ulrich et al. 1993) and light (Findlay et al. 1993, Fuller et al. 2004, Ulrich et al. 1993). In these streams, pH has been shown to affect periphyton biomass and fungal densities (Hall et al. 1980). Light is necessary for stream autotrophs, but light and correlated increases in temperature can also increase the growth, density, and enzymatic activity of heterotrophic microbes co-occurring with autotrophs (Romani and Sabater 1999, Ylla et al. 2009). Studies on biofilms as a whole, quantifying both autotrophic and heterotrophic components, are lacking at this site, as are studies on top-down controls (but see Bernhardt and Likens 2004, Findlay et al. 1993).

Our goal was to explore the influence of bottom-up and top-down controls on biofilm biomass across the Hubbard Brook valley. We predicted that light, nutrients, physical habitat structure, and stream chemistry would be important bottom-up variables influencing biofilm biomass. Top-down control of biofilm biomass at the HBEF may occur directly through its primary consumers, benthic macroinvertebrates (Hall et al. 2001), or indirectly through stream salamanders, which prey on benthic macroinvertebrates, potentially releasing biofilm from direct limitation by macroinvertebrates (Burton 1976). If biofilm biomass is controlled by top-down predation by benthic macroinvertebrates, we would expect biofilm biomass to decline with increasing benthic macroinvertebrate biomass, our index of the macroinvertebrate community. However, other relationships are also possible depending on the relative effects of bottom-up and top-down controls on the macroinvertebrates themselves (Borer et al. 2005), and the degree to which aggregate benthic macroinvertebrate biomass reflects taxa that use biofilm as a resource (Merritt and Cummins 1996). We also tested for effects of bottom-up and top-down controls on macroinvertebrate biomass. Specifically, we wanted to assess whether salamander occupancy influenced the immediate consumers of biofilm, benthic macroinvertebrates. Alternatively, benthic macroinvertebrate biomass may be found to be positively correlated with biofilm biomass, suggesting that bottom-up control of benthic macroinvertebrates is occurring.

Methods

Selection of study area and study reach

To assess bottom-up and top-down controls of biofilm biomass, we sampled 20 fishless study reaches throughout the stream network at the HBEF. The HBEF is an oligotrophic watershed located in the White Mountains Region of central New Hampshire (43°56′N, 71°45′W). Field sampling took place between 1 July and 10 September 2016. The HBEF is part of the Long-Term Ecological Research Network (LTER). The top predators at our sampling reaches are 2 stream salamanders, *Gyrinophilus porphyriticus* (Green) (Spring Salamander) and *Eurycea bislineata* (Green) (Northern Two-lined Salamander) (Burton 1976). A third species, *Desmognathus fuscus* (Rafinesque) (Northern Dusky Salamander), also occurs in our study streams but was not encountered during our surveys. These salamanders have aquatic larvae that prey on benthic macroinvertebrates, and Spring Salamander larvae and adults also consume Northern Two-lined Salamanders and terrestrial insects that fall on the stream's surface (Burton 1976, Lowe et al. 2005, Petranka 1998). The forest at the HBEF is dominated by *Acer saccharum* Marshall (Sugar Maple), *Fagus grandifolia* Ehrhart (American Beech), and *Betula alleghaniensis* Britton (Yellow Birch) (Likens 2013). The HBEF streams also tend to be heterotrophic, with most carbon entering the streams through allochthonous inputs (Fisher and Likens 1973, Mayer and Likens 1987). These streams are nutrient poor, with average fall concentrations of ammonium, nitrate, and phosphate values of 0.01, 0.03 and 0.003 mg/L, respectively (Likens and Buso 2006).

We randomly selected study reaches 10 m in length from a list of stream monitoring sites across the entire stream network at the HBEF (Likens and Buso 2006). We used a vector file of Hubbard Brook hydrography (http://data.hubbardbrook.org/gis/; accessed 24 August 2014) to determine the distance between selected study reaches in QGIS 2.14.3-Essen (QGIS Development Team 2016). To increase independence among study reaches, we rejected study reaches that were less than 200 m from another study reach. We also rejected those that were within designated experimental watersheds where intensive in-stream sampling is discouraged. Finally, because we were specifically interested in controls on biofilm biomass in fishless headwater streams, we rejected reaches where fish have been detected. We based fishless status of study reaches on Warren et al. (2008). We confirmed fish absence from study reaches by placing 3 minnow traps (2.54-cm opening; model 0822711271, Frabill, Jackson, WI) in study reaches for 24 hours prior to conducting surveys. We randomly chose replacement reaches until 20 study reaches were selected (Fig. 1). We sampled these study sites in random order throughout the field season (July–September) to mitigate the effects of stochastic variation in stream discharge and other stochastic environmental effects on biofilm accrual. The sampling period was, however, deliberately timed to coincide with the period of hydrologic stability at the HBEF. Hydrology of HBEF streams is characterized by high spring discharge due to melting snow, and high discharge events throughout the year associated with isolated storms. Base flow conditions usually occur from mid-July through mid-September. We collected all field data for a study reach on the sampling day.

Several of the randomly selected study reaches ($n = 11$) were located along the same stream channel. To ensure that data from these study reaches were independent, we ran t-tests comparing the pH, conductivity, and nutrient data between 2 study reaches located on the same stream channel and 2 randomly chosen study reaches. We conducted these comparisons for all 11 study reaches of interest. All t-test were not significant ($P > 0.20$), indicating that paired study reaches were not more similar than non-paired study reaches.

Biofilm sampling

At each study reach, we collected biofilm from 3 cobbles at each of 3 distances from the downstream end of the reach: 0, 5, and 10 m. At some reaches, 3 cobbles were not available at each distance, so we collected biofilm from all available cobble (total number of cobbles scraped per study reach ± SE = 8 ± 0.5). We selected only cobbles that were submerged in the stream and free of moss and chose individual rocks haphazardly. On each cobble, we scraped an area of 23.8 cm^2 using a razorblade. We placed the collected biofilm on ice in the dark and froze it within 5 hrs (Kilroy et al. 2013). Samples were kept frozen until they were ashed and weighed using Standard Method 2540E (American Water Works Association and Water Environment Federation 2005). We calculated biomass of biofilm at each reach as the average of values at 0, 5, and 10 m. Values for each location were mean biomass of biofilm in grams per m^2, calculated from the area-standardized samples of replicate cobbles at each distance.

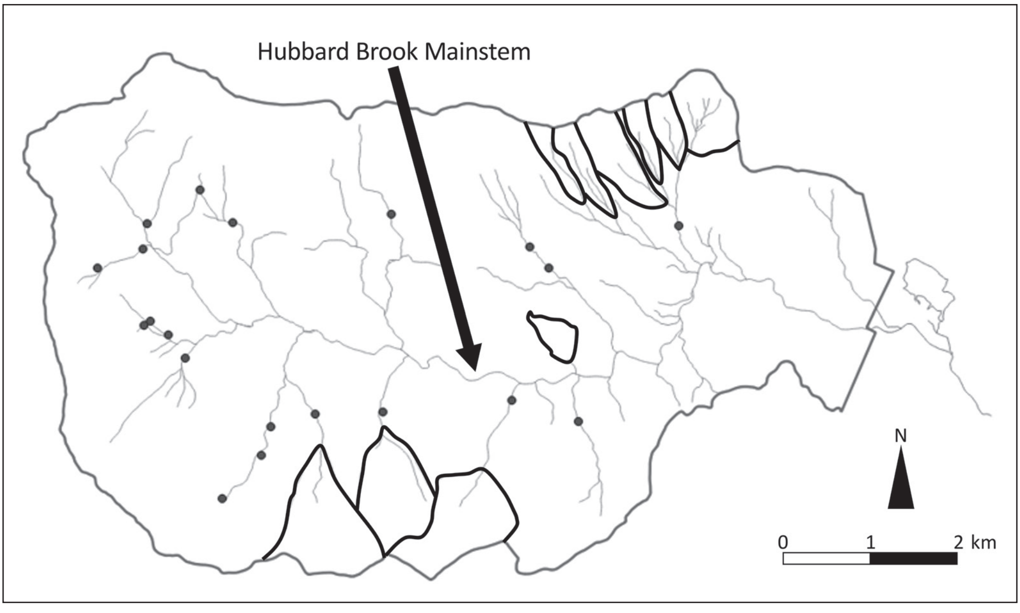

Figure 1. Map of reaches where biofilm was collected at the Hubbard Brook Experimental Forest, NH ($n = 20$). Reaches were visited between 1 July 2016 and 10 September 2016. Experimental watersheds are outlined and were excluded from sampling.

Bottom-up controls

We predicted that light, nutrients, physical habitat structure, and stream chemistry would be important bottom-up controls of biofilm and macroinvertebrate biomass. We used aspect and canopy cover as proxies for light availability at the water surface. Aspect is known to be an important factor regulating growth in both terrestrial and aquatic plants due to its effects on light, transpiration, and temperature (Cantlon 1953). Specifically, in the Northern hemisphere, a southern aspect is known to provide more light and induce more growth than a northern aspect when water is not limiting (Holland and Steyn 1975). We determined aspect (southern or northern) in relation to the Hubbard Brook mainstem, which flows roughly east to west through the center of the HBEF (Fig. 1). Whereas aspect influences light availability at the scale of an entire watershed, canopy cover provides a more proximate index of light availability to the study reach. We measured canopy cover using a spherical convex densitometer (Forestry Suppliers Inc., Jackson, MS; Lemmon 1956). We took 6 measurements at 0, 5, and 10 m from the downstream end of each study reach. At each distance, we took 4 measurements in the center of the stream (facing upstream, downstream, the right bank, and the left bank) and 2 measurements while standing on the banks (1 on the right and 1 on the left bank). We used the average of the 18 canopy cover measurements from a study reach in our analysis (Table 1; Plotnikoff and Wiseman 2001). Ideally, rather than relying on these indirect indices of light availability and temperature, we would have measured photosynthetically active radiation (PAR) and water temperature continuously throughout the growing season. However, we did not have the funding to purchase remote light and temperature loggers for all 20 study reaches.

The specific nutrients that we predicted would affect stream biofilm and macroinvertebrate biomass were nitrate (NO_3^-), phosphate (PO_4^{3-}), calcium (Ca^{2+}), magnesium (Mg^{2+}), dissolved inorganic carbon (DIC), silicon dioxide (SiO_2), and dissolved organic carbon (DOC). We obtained values for each of these nutrients for each of our study reaches, in milligrams per liter, from Likens and Buso (2006) (Table 1), a uniquely intensive study where streamwater chemistry was measured at 100-m intervals in all streams of the Hubbard Brook Valley in May–July of 2001. There has been a steady decline in stream water concentrations of NO_3^-, PO_4^{3-}, Ca^{2+}, Mg^{2+} and a concurrent increase in stream pH across the HBEF since the 1960s (Fuss et al. 2015, Likens et al. 1996). However, as the long-term data sets from Hubbard Brook experimental watersheds show, these trends have been gradual, and all measurements of streamwater chemistry show within- and among-year variation that far exceeds these temporal trends (Fuss et al. 2015, Likens 2013). In using the valley-wide data from Likens and Buso (2006), we are not assuming that conditions in 2016 were identical to those in 2001, but we are assuming that relative differences in conditions among sites have remained consistent across years. This assumption is supported by the long-term streamwater chemistry data from the Hubbard Brook experimental watersheds (Likens 2013), and by Likens and Buso (2006), who repeated valley-wide sampling in October–December of 2001 and found that differences among sites were similar to those found in May–July of the same year.

We expected many of these nutrient variables to be correlated, which can lead to spurious results in regression analyses (Graham 2003). Therefore, we used principal component analysis (PCA) to reduce the dimensionality of our stream nutrient data to 1 independent variable (nutrient PC 1). In PCA, the original variables are replaced by an equal number of principal components, which are each a linear combination of the original variables (Jolliffe and Cadima 2016). Each principal component is uncorrelated with any other principal component and the components are ordered by the amount of variation in the data they explain. Therefore, the effect of individual nutrients on biofilm biomass is obscured by PCA. However, we may associate variables and principal components, provided the component loading's (CL) magnitude is relatively large (Huryn et al. 2002).

The physical habitat variables that we predicted would influence biofilm and macroinvertebrate biomass were elevation (m), stream width (m), percent total wood, mesohabitat, and substrate. We determined the elevation of each of our study reaches using data from Likens and Buso (2006). We measured stream width as the bankfull stream width at each study reach, which we averaged from measurements at 0, 5, and 10 m along the reach. We visually estimated the percent of stream bed covered by coarse woody debris and the percent of each stream mesohabitat (i.e., pools, rifles, runs, or cascades) at each study reach (Table 1). Stream mesohabitat type are commonly used to describe covariation in channel gradient and water-flow conditions along streams (Montgomery and Buffington 1998). We identified pools

Table 1. Min–max, mean, and standard errors (SE) for variables used in principal component analysis (PCA) to assess bottom-up and top-down controls on biofilm in 20 headwater stream reaches in the Hubbard Brook Experimental Forest, NH. We ran 3 separate PCA analyses: 1 each that summarized nutrients, substrate features, and the stream mesohabitat. Nutrient data from Likens and Buso (2006).

Variable	PCA	Min–max	Mean	SE
DOC (mgL^{-1})^{AB}	Nutrients	1.28–7.54	2.94	0.35
DIC (mgL^{-1})^{AC}	Nutrients	30–222	64.70	9.53
Ca^{2+} (mgL^{-1})	Nutrients	0.50–1.42	1.00	0.05
NO_3^- (mgL^{-1})^{A}	Nutrients	0.01–0.23	0.04	0.01
PO_4^{3-} (mgL^{-1})	Nutrients	0.0040–0.0005	0.0012	0.0002
Mg^{2+} (mgL^{-1})	Nutrients	0.13–0.61	0.34	0.02
SIO_2 (mgL^{-1})	Nutrients	3.2–7.8	5.89	0.029
% sand^{D}	Substrate	0–48	5.50	2.36
% gravel	Substrate	2–26	11.05	1.46
% cobble	Substrate	2–32	17.85	1.56
% boulder	Substrate	11–88	54.05	3.87
% bedrock^{D}	Substrate	0–51	11.50	2.74
% riffle	Mesohabitat	0–65	30.25	4.34
% run	Mesohabitat	10–85	38.25	4.91
% pool	Mesohabitat	5–60	25.00	3.26
% cascade	Mesohabitat	0–20	6.50	1.26

[A] Inverse taken to meet the assumption of normality.
[B] Dissolved organic carbon.
[C] Dissolved inorganic carbon.
[D] Variable was $\log_{10}$ transformed to meet the assumption of normality

by evidence of scour caused by obstruction, blockage, merging of flows, or constriction; runs by low gradient and laminar flow; riffles by moderate gradient and turbulent flow; and cascades by high gradient and highly turbulent flow. Because study reaches were 10 m in length, we were able to visually survey entire reaches from several points within the reach, allowing for consistent estimates of percent coverages of woody debris and mesohabitat types.

We also conducted a Wolman pebble count to characterize stream substrate at each study reach (Wolman 1954, Yan et al. 2005). Briefly, for the Wolman pebble count, we recorded substrate type (i.e., sand, gravel, cobble, boulder, bedrock) every meter in transects that were perpendicular to stream flow and covered the bankfull width of the stream. Moving upstream, we repeated this process until we recorded 100 measurements or had traversed the entire study reach. For each study reach, we then calculated the percent of each substrate type. As with nutrients, we expected the percent of each mesohabitat type and percent of each stream substrate type at a study reach to be autocorrelated and used principal component analysis to reduce these data sets to single variables (mesohabitat PC 1, substrate PC 1). We then used mesohabitat PC 1 and substrate PC 1 in our analyses.

The additional stream chemistry variables predicted to influence biofilm biomass and macroinvertebrates were water conductivity and pH. Both conductivity and pH can alter community composition of biofilms (Lear et al. 2009, Ledger and Hildrew 2001, Wilhelm et al. 2013), and pH also alters the community composition of benthic macroinvertebrate consumers (Ledger and Hildrew 2005). As with nutrients, we obtained stream-chemistry data for each of our stream reaches from Likens and Buso (2006).

Top-down controls

We predicted that benthic macroinvertebrate biomass and stream salamander occurrence would be important top-down variables influencing biofilm biomass. We acknowledge that macroinvertebrate biomass is a course measure, but—given that the large number of study reaches limited us to 1 round of sampling—we felt that it would better represent top-down predation pressure than abundance because it also accounts for body size and energetic demand (Borer et al. 2005). The large number of samples also precluded identifying all individuals, so we were unable to subsample only those taxa known to use biofilm. Furthermore, identifying these taxa in the Hubbard Brook streams is difficult; although macroinvertebrate communities there are dominated by taxa that use allochthonous inputs from the surrounding forests (Hall et al. 2001), these taxa can rely on autochthonous carbon for growth (Mayer and Likens 1987, McCutchan and Lewis 2002). Additionally, total macroinvertebrate biomass includes predacious species that feed on biofilm consumers, thus accounting for direct and indirect effects of biofilm on the macroinvertebrate community (Borer et al. 2005). We assumed, therefore, that total biomass was an accurate and conservative index of top-down pressure by macroinvertebrates.

To measure benthic macroinvertebrate biomass, we obtained samples from 0, 5, and 10 m at each study reach. We collected samples by disturbing substrate within a

0.09-m^2 quadrat in riffle habitats for 2 minutes and collecting benthic macroinvertebrates in a 800 µm × 900 µm mesh D-frame net placed immediately downstream of the sampling quadrat. When a riffle was not present, we sampled a run. We filtered samples through 2 nested sieves of mesh size 10 mm^2 and 1 mm^2 and placed all benthic macroinvertebrates in the 1-mm^2 sieve on ice in the field (Angradi 1996). Upon return to the lab, we froze macroinvertebrates within 48 hours. After thawing, we dried samples at 65 °C for 72 hours, cooled them in a desiccator, then weighed them to the nearest 0.0001 gram. We recorded biomass of benthic macroinvertebrates at a study reach as the average of total biomass at 0, 5, and 10 m.

To determine salamander occupancy at a reach, we used both cover-controlled and area-constrained survey methods. We modified cover-controlled active survey methods from Lowe and Bolger (2002). Specifically, we flipped 3 cobbles (64–256 mm in length by the longest dimension), located either within the stream or along the bank, every meter for a total of 30 rocks per reach. We collected all Spring Salamander individuals revealed or flushed by the current in an aquarium dip-net. We conducted 1 area-constrained survey at 0, 5, and 10 m. We placed a 0.38-m^2 quadrat on the stream bed, removed all cobble within the quadrat, and collected all Spring Salamander and Northern Two-lined Salamander individuals observed. We constrained area-controlled searches to 60 minutes and released salamanders at their collection site. We considered a reach occupied by Spring Salamanders or Northern Two-lined Salamanders if 1 or more individuals of that species were detected using these survey methods. Because we detected Northern Two-lined Salamander larvae at all but 2 of our study reaches, we were not able to use Northern Two-lined Salamander occupancy in our analyses.

Statistical analyses

To assess the effects of bottom-up and top-down variables on biofilm biomass and macroinvertebrate biomass at the HBEF, we used multiple linear regression with full stepwise model selection (Kleinbaum et al. 2013). The bottom-up variables included in our models were aspect, canopy cover, nutrient PC 1, elevation (m), stream width (m), percent total wood, mesohabitat PC 1, substrate PC 1, pH, and conductivity (Table 2). The top-down variables included in the biofilm model were benthic macroinvertebrate biomass and occupancy of Spring Salamanders (Table 2). For the macroinvertebrate model, Spring Salamander occupancy was the only top-down variable. We included date as an additional variable in the model-selection process to account for possible temporal variation in biofilm and macroinvertebrate biomass over the sampling period. When necessary, independent variables were transformed to meet the assumption of normality (Tables 1, 2). We tested for autocorrelation of independent variables using Pearson's correlation analysis prior to running the multiple stepwise linear regression. Explanatory variables were not autocorrelated ($r < 0.7$).

The full stepwise procedure utilized both forward selection and backwards elimination. We used the Akaike information criterion modified for small sample sizes (AIC_c) to select the most parsimonious model (i.e., with the lowest AIC_c score) from all candidate models (Burnham and Anderson 2002). We calculated the difference in AIC_c scores for each of the top models, as well as their likelihoods and Akaike weights. If the top 2 models differed in AIC_c weight by ≤ 2.0, we used a likelihood ratio test to identify the most parsimonious model (Burnham and Anderson 2002, Johnson and Omland 2004). We include partial *P*-values for each of the variables in the top model to provide information on relative importance, but recognize that *P*-values are not, strictly speaking, applicable to models chosen using an information-theoretic approach (Burnham and Anderson 2002). We also examined univariate regressions of each of the explanatory variables in the top model against biofilm biomass to assess the strength and directionality of individual effects. All statistical analysis were conducted in R version 3.4.3 (R Core Team 2017). Principal components analyses were conducted using the function 'prcomp()' built into the 'stats' package in R. Multiple regression analyses were conducted using the 'StepAICc' script (Venables and Ripley 2002).

Results

Predictors of biofilm biomass

Nutrient PC 1 explained 43.5% of the variation in stream nutrients. The component loadings for nutrient PC 1 were Ca^{2+} (CL = 0.50), DIC^{-1} (-0.45), Mg^{2+} (0.43), SiO_2 (0.22), $(NO_3^-)^{-1}$ (-0.05), DOC^{-1} (0.38), and PO_4^{3-} (-0.41). We used the inverse of DIC, DOC, and in NO_3^- the principal component analysis. Therefore, nutrient PC 1 was primarily positively associated with Ca^{2+}, DIC and Mg^{2+}, and negatively associated with PO_4^{3-} Mesohabitat PC 1 explained 48% of the variation in percent of stream mesohabitat composed of cascades, riffles, pools, and runs among study reaches. The component loadings for mesohabitat PC 1 were percent cascade (CL = 0.48), percent riffle (0.45), percent pool (0.26) and percent run (-0.70). Substrate PC 1 explained 48% of the variation in percent of stream substrate composed of

Table 2. Min–max, mean, and standard errors (SE) of variables used in stepwise multiple linear regression to assess bottom-up and top-down controls on biofilm in 20 headwater stream reaches in the Hubbard Brook Experimental Forest, NH.

Variable	Min–max	Mean	SE
Biofilm (gm^{-2})	0.88–2.82	1.90	0.13
Benthic macroinvertebrates (gm^{-2})^A	0.04–0.44	0.18	0.03
Date, Julian	183–254	215.10	5.44
pH	4.66–6.68	5.67	0.11
Conductivity ($uScm^{-1}$)	12.9–20.1	15.75	0.53
Canopy cover	88.77–96.77	92.64	0.50
Elevation (m)	402–703	581.40	14.90
Bankfull width (m)	0.8–6.7	3.62	0.30
% total wood^A	2.5–35.0	13.38	2.32
Nutrient PC 1	-3.45–3.54	0.00	0.39
Substrate PC 1	-2.89–3.43	0.00	0.35
Mesohabitat PC 1	-2.57–2.33	0.00	0.31

^{A}Variable was log_{10} transformed to meet the assumption of normality.

sand, gravel, cobble, boulder, or bedrock among study reaches. The component loadings for substrate PC 1 were percent gravel (CL = 0.60), percent sand (0.44), percent cobble (0.36), percent bedrock (-0.14), and percent boulder (-0.54).

The top 2 models predicting biofilm biomass at the HBEF selected by stepwise multiple linear regression had similar AIC_c weights ($\Delta AIC_c < 2.0$; Table 3). A likelihood ratio test comparing these 2 models was not significant ($P = 0.06$), indicating that a model with fewer parameters was marginally more parsimonious. This model included aspect, canopy cover, nutrient PC 1, and pH as explanatory variables. The top model explained 46% of the total variation in biofilm biomass (Table 3). Aspect, canopy cover, nutrient PC 1, and pH were also included in the 3 next highest-ranked models (Table 3). Of these, aspect, canopy cover, and pH had partial *P*-values less than 0.05 (Table 4). Biofilm biomass was negatively associated with canopy cover, suggesting that light increased biofilm biomass. Streams with a southern aspect also tended to have more biofilm, further emphasizing the importance of light for biofilms at the HBEF. The positive correlation of biofilm biomass and nutrient PC 1, given the weighting of nutrient PC 1, indicates that biofilm biomass is positively associated with Ca^{2+}, DIC and Mg^{2+}, and negatively associated with PO_4^{3-} . Biofilm biomass was negatively correlated with stream pH, indicating that biofilm biomass increased as streams became more acidic. The 2 potential top-down controls of

Table 3. Summary of multiple regression models for biofilm biomass in 20 headwater stream reaches in the Hubbard Brook Experimental Forest, NH, selected using stepwise regression and AIC_c scores to determine model rank. Δ_i are the AIC_c differences and w_i are the AIC_c weights. Due to a small AIC_c difference between the top 2 models, a likelihood ratio test was used to determine the most parsimonious model. The top model as determined by the likelihood ratio test is designated by an asterisk (*). Nutr = nutrient PC 1, canopy = canopy cover, meso = mesohabitat PC 1, cond = conductivity, and bankfull = bankfull width.

Model	Rank	AIC_c	Δ_i	Likelihood $[L(g_i\|x)]$	w_i	P	Adjusted R^2
pH, nutr, canopy, aspect	1*	36.59	0.00	1.00	0.607	0.009	0.46
pH, nutr, canopy, aspect, meso	2	37.82	1.23	0.54	0.328	0.008	0.51
pH, nutr, canopy, aspect, meso, cond	3	41.21	4.62	0.10	0.060	0.010	0.54
pH, nutr, canopy, aspect, meso, cond, elevation	4	46.36	9.77	0.01	0.005	0.010	0.54
pH, nutr, canopy, aspect, meso, cond, elevation, bankfull	5	50.53	13.90	0.00	0.001	0.010	0.59

Table 4. Estimates from the top model of biofilm biomass selected by stepwise selection including variable coefficients (β), coefficient standard errors (SE), *t*-statistic (*t*), and partial *P*-values (*P*). This study was conducted in 20 headwater stream reaches in the Hubbard Brook Experimental Forest, NH.

Variable	β	SE	t	P
(Intercept)	26.19	5.70	5.60	0.000
pH	-0.82	0.28	-2.91	0.011
Nutrient PC 1	0.14	0.76	1.89	0.078
Canopy cover	-0.21	0.05	-4.07	0.001
Aspect (south)	0.49	0.22	2.21	0.044

biofilm, benthic macroinvertebrate biomass and Spring Salamander occupancy, were not included in any of the top models. Again, because Northern Two-lined Salamanders were detected at all but 2 of our study reaches, we were not able to use Northern Two-lined Salamander occupancy in this analysis.

None of the univariate regressions of the predictor variables in the top model for biofilm biomass were significant at $P < 0.05$. However, the univariate regression of canopy cover was nearly significant ($P = 0.052$) and explained 15% of the total variation in biofilm biomass (Fig. 2).

Predictors of benthic macroinvertebrate biomass

The top 2 models predicting benthic macroinvertebrate biomass selected by stepwise multiple linear regression also had similar AIC_c weights ($\Delta AIC_c < 2.0$; Table 5). A likelihood ratio test comparing these top 2 models was not significant ($P = 0.08$), indicating that the model with fewer parameters was most supported. That model included date and substrate PC 1 and explained 36% of the variation in benthic macroinvertebrate biomass (Table 5). Date had a P-value less than 0.05, but substrate did not (Table 6). Benthic macroinvertebrate biomass decreased over the sampling period (from 1 July to 10 September 2016) and with increasing substrate PC 1 (i.e., with more gravel and fewer boulders).

Discussion

Despite the dominance of heterotrophic microorganisms in biofilms of the heavily shaded headwater streams of the HBEF (Burton et al. 1988, Webster et al. 2003), light availability was a strong predictor of biofilm biomass. Specifically, we interpreted the presence of both aspect and canopy cover in our top model,

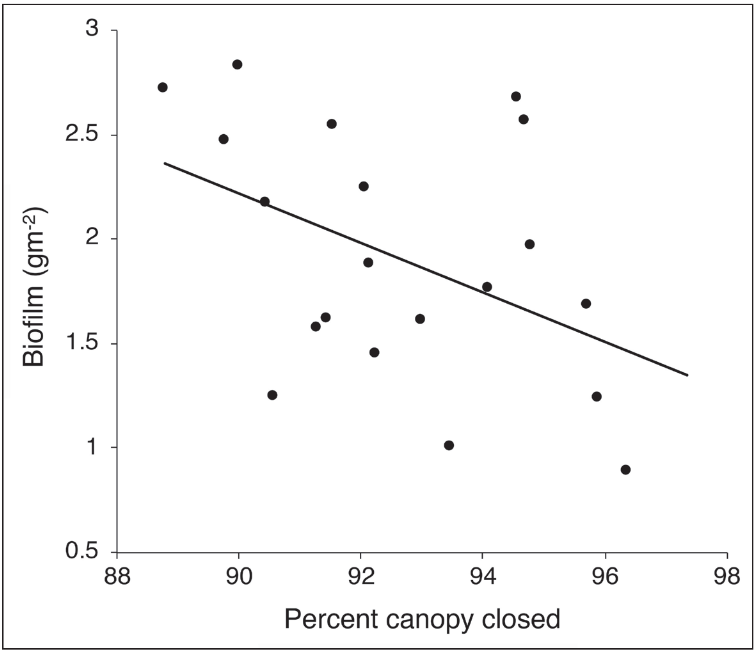

Figure 2. Post-hoc analysis of biofilm (grams AFDM per meter squared) versus percent canopy closed for all reaches ($n = 20$). Univariate linear regression showed this relationship to be marginally significant ($P = 0.052$), explaining 15% of the variation in biofilm biomass among reaches at the Hubbard Brook Experimental Forest, NH.

and 4 sequential models, as reflecting the importance of light to biofilm biomass (Table 3). Southern aspect is associated with greater light availability in the Northern hemisphere (Galicia et al. 1999, Geiger et al. 2009), and was correlated with higher biofilm biomass in our top model. Canopy cover was high in all of our study reaches and varied from 88.8% to 96.8% (mean = 92.6%; Table 2), yet still had a strong relationship with biofilm biomass. It is also possible that canopy cover and aspect affected biofilm biomass through changes in stream temperature (Swift and Messer 1971, Wilkerson et al. 2006, Williamson et al. 2016) or minor variation in leaf-litter inputs to streams (Martínez et al. 2017).

Given previous studies showing that biofilms in the HBEF are dominated by heterotrophic members (Webster et al. 2003), the strong influence of light on biofilm biomass may indicate that heterotrophic bacteria and fungi are benefiting from algal carbon fixation (Romani and Sabater 1999, Ylla et al. 2009) or from the structural scaffolding that algae—and diatoms in particular—bring to stream biofilms (Battin et al. 2016, Romani et al. 2004). This interpretation is supported by evidence that bacterial counts increase seasonally with increasing algal and cyanobacteria counts in the HBEF (Burton et al. 1988), but conflicts with the findings of Findlay et al. (1993), who found no evidence for algae–bacteria linkages in HBEF streams. The importance of light for stream biofilm biomass is also consistent with previous studies at the HBEF showing that algae increased in streams after clearcutting (Haack et al. 1988, Noel et al. 1986), that the main Hubbard Brook has higher algal concentrations than its shadier counterpart, Bear

Table 5. Summary of the top 5 models for benthic macroinvertebrate biomass selected using stepwise regression based on AIC$_c$ scores. Δ_i are the AIC$_c$ differences and w_i are the AIC$_c$ weights. Due to a small AIC$_c$ difference between the top 2 models, a likelihood ratio test was used to determine the best supported model. The top model as determined by the likelihood ratio test is designated by an asterisk (*). This study was conducted in 20 headwater stream reaches in the Hubbard Brook Experimental Forest, NH. Sub = substrate PC 1, canopy = canopy cover, cond = conductivity, and bankfull = bankfull width.

Model	Rank	AIC$_c$	Δ_i	Likelihood $[L(g_i \vert x)]$	w_i	P	Adjusted R^2
Sub, date	1*	7.22	0.00	1.00	0.501	0.008	0.36
Sub, date, canopy	2	7.67	0.45	0.80	0.400	0.008	0.42
Sub, date, canopy, cond	3	10.65	3.43	0.18	0.090	0.014	0.42
Sub, date, canopy, cond, aspect	4	15.31	8.09	0.02	0.009	0.033	0.38
Sub, date, canopy, cond, aspect, bankfull	5	20.50	13.28	0.00	0.001	0.060	0.36

Table 6. Summary statistics for the final model of benthic macroinvertebrate biomass selected by stepwise AIC$_c$, including variable coefficients (β), coefficient standard errors (SE), t-statistic (t), and partial P-values (P). This study was conducted in 20 headwater stream reaches in the Hubbard Brook Experimental Forest, NH.

Variable	β	SE	t	P
(Intercept)	0.40	0.51	0.77	0.450
Substrate PC 1	0.07	0.04	1.84	0.083
Date	-0.01	0.00	-2.41	0.027

Brook (Hall et al. 2001), and that shading can reduce periphyton (Findlay et al. 1993, Fuller et al. 2004, Ulrich et al. 1993).

The positive correlation of biofilm biomass with nutrient PC 1 suggests that nutrients used for structural support may be more important to biofilm production at the HBEF than nitrogen and phosphorus. Ca^{2+}, DIC, and Mg^{2+} were most positively associated with nutrient PC 1 (component loadings for Ca^{2+}, $(DIC)^{-1}$, and Mg^{2+} were 0.50, -0.45, and 0.43, respectively). Calcium and magnesium are important for biofilm adhesion and the stability of the EPS matrix, which makes up the scaffolding of biofilm (Flemming et al. 2016, Geesey et al. 2000, Song and Leff 2006). Removal of these cations results in dissolution of biofilms (Banin et al. 2006). DIC can be important for the autotrophic components of biofilm, particularly in acidic conditions (Fairchild and Sherman 1993, Vinebrooke 1996). PO_4^{3-} had the strongest negative association with nutrient PC 1, while nitrogen (which was inverse transformed for analysis) had a slightly positive association (component loadings for PO_4^{3-} and $(NO_3^-)^{-1}$ were -0.41 and -0.05, respectively). These findings are consistent with previous studies at the HBEF, which found that nitrogen and phosphorus had neutral or inhibitory effects on periphyton biomass (Bernhardt and Likens 2004, Ulrich et al. 1993). These results emphasize the importance of considering a wide variety of nutrients when assessing controls on biofilm growth (Kaspari and Powers 2016).

Biofilm biomass was negatively correlated with pH (Table 4), even though the autotrophic and heterotrophic components of biofilm respond to pH differently, raising questions about the emergent properties of biofilms at the HBEF. The streams we sampled were acidic (mean pH = 5.67; Table 2) due in part to the legacy of acid rain (Johnson et al. 1981, Likens et al. 1996). However, the lowest pH we encountered (pH = 4.66) was higher than an acid-addition treatment at the HBEF (pH = 4.3) that had no effect on chlorophyll-*a* concentrations (Ulrich et al. 1993). Additionally, periphyton biomass at the HBEF increased when a stream was artificially maintained at a pH of 4.0 (Hall et al. 1980). This result may be due to the dominance of acid-tolerant diatom and algal species (Ulrich et al. 1993) or the ability of biofilms to alter internal pH gradients, both through excretions and by the creation of a diffusion boundary layer (Cornwall et al. 2014, Vroom et al. 1999). However, experimental reductions of pH in streams at the HBEF resulted in lower biofilm bacterial and fungal densities (Haack et al. 1988, Hall et al. 1980), suggesting that if biofilm is buffering pH, the heterotrophic components of biofilm do not seem to benefit from this buffering. Overall, these findings suggest that the negative correlation between biofilm biomass and pH we observed resulted from positive effects of low pH on autotroph biomass, which outweighed negative effects on heterotrophic microbes.

We did not find support for top-down controls on biofilm biomass despite evidence of top-down controls on periphyton biomass in other contexts (Bourassa and Cattaneo 1998, Feminella and Hawkins 1995, Hillebrand 2002, Lamberti 1996). Neither macroinvertebrate biomass nor salamander occurrence was present in top models (Table 3). Because we collected biofilm from cobble, detecting direct and indirect top-down effects would have depended primarily on the behavior or density of benthic macroinvertebrates found on cobbles, including members of the scraper functional feeding group and their predators (Merritt and Cummins 1996). However, previous studies have found that scrapers make up ≤13% of the benthic macroinvertebrate assemblage of streams at the HBEF (Chadwick and Huryn 2005, Hall et al. 2001), which may make these top-down effects difficult to detect. Future studies should look at biofilm biomass on both cobbles and leaves to assess top-down effects of scraping and shredding invertebrates. Shredders feed on biofilm attached to leaves and make up 34–50% of the benthic macroinvertebrate assemblage in streams at the HBEF (Chadwick and Huryn 2005, Hall et al. 2001). Because we did not identify the functional groups of macroinvertebrates in our samples, we cannot address these hypotheses with our data. Additionally, it is possible that the HBEF's nutrient-poor streams and low levels of primary and secondary production (Chadwick and Huryn 2005, Likens 2013) do not support the densities of benthic macroinvertebrates needed to exert top-down pressure on biofilms.

The community composition of salamanders in streams at the HBEF may also explain why we did not see top-down effects of salamanders on biofilm biomass. There is evidence that salamanders can decrease abundances of stream macroinvertebrates (Atlas and Palen 2014, Keitzer and Goforth 2013, Progar and Moldenke 2002), but salamander occupancy was not present in our top models of benthic macroinvertebrate biomass (Table 5) or biofilm biomass (Table 3). Importantly, our models included only occupancy of Spring Salamanders because the smaller salamander species, the Northern Two-lined Salamander, was only absent from 2 study reaches. In a posteriori analyses, we ran the multiple regression analyses of biofilm biomass and benthic macroinvertebrate biomass with estimates of Spring Salamander and Northern Two-lined Salamander densities (individuals / m^2) derived from our surveys as independent variables, instead of occupancy. Including these variables did not change the top models (Tables 3, 5). A separate experimental study examining top-down effects of Spring Salamanders and Northern Two-lined Salamanders on benthic macroinvertebrates, both alone and together, found that only Spring Salamanders affected benthic macroinvertebrate abundances, and only when it did not co-occur with Northern Two-lined Salamanders (Bayer and Lowe, in press). This experiment indicated that when the 2 species occur together, as was the case at all reaches where we detected Spring Salamanders, Spring Salamanders feed primarily on Northern Two-lined Salamanders, not benthic macroinvertebrates.

Our findings are consistent with the hypotheses that (1) light, nutrients, and pH regulate biofilm biomass in streams at the HBEF, (2) the auto- and heterotrophic components of biofilm respond to these variables differently, and (3) emergent properties of biofilms, such as structural stability or pH tolerance, determine biomass accrual. Similarly, the lack of significance of univariate regression analyses suggests that multiple bottom-up controls of biofilm biomass must be considered simultaneously for the contribution of any 1 variable to be evident. Although top-down controls on biofilm have been shown in other systems (Feminella and

Hawkins 1995, Hillebrand 2002, Winkelmann et al. 2014), their absence in our models suggests that controls of stream biofilm biomass at the HBEF are primarily bottom-up. Biofilms of headwater streams are frequently the first sites of terrestrial nutrient uptake and transformation, determining both the local availability and downstream export of these nutrients, while also providing an important source of in situ productivity supporting stream food webs. To fully understand the role of stream biofilms in nutrient cycling and stream food webs, we recommend that future studies isolate bottom-up and top-down effects on autotrophic and heterotrophic components of biofilm separately.

Acknowledgments

We thank A. McTaggart for field assistance, and L. Eby, N. Rodenhouse, J. Maron, C. Filardi, and R. Hauer for comments on this manuscript. This research was funded by the National Science Foundation (DEB-1050459, DEB-1114804, DEB-1655653, and DEB-1637685) and was conducted under Montana State Institutional Care and Use Protocol # 003-14WLDBS-012714. This manuscript is a contribution to the Hubbard Brook Ecosystem Study. The Hubbard Brook Experimental Forest is operated and maintained by the Northeastern Forest Research Station, USDA Forest Service, Newtown Square, PA.

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