Substrate-specific biofilms control nutrient uptake in experimental streams

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Abstract: Substrate heterogeneity and biofilm colonization in streams vary across both time and space, but their relative contribution to reach-scale nutrient uptake is difficult to partition. We performed multiple short-term nutrient additions over a 4-mo colonization sequence in 4 small, groundwater-fed, experimental streams. We quantified the influence of substrate size (pea gravel vs cobble) and heterogeneity (alternating sections vs well mixed) on the uptake of NH_4^+, NO_3^-, and soluble reactive P (SRP) and transient storage properties. In general, the effect of benthic substrate on uptake velocity (v_f) and areal nutrient uptake (U) were inversely related to substrate size, and both metrics were highest in the stream lined with pea gravel, lowest in cobble, and intermediate in streams with alternating and mixed substrates. Substrate trends were consistent among solute types, but the magnitude of uptake differed. Uptake generally was higher for NH_4^+ than for NO_3^- and SRP in these open-canopy systems. Algal biomass controlled temporal patterns of nutrient uptake but reduced exchange of water between the stream channel and transient storage zone (k_1) such that k_1 decreased as nutrient uptake increased. Our results uniquely demonstrate that substrate heterogeneity and substrate-specific biofilms interact to influence biogeochemical cycling in streams, with implications for the role of substrate in restoring ecosystem function in impaired systems.
Key words: nutrient uptake, benthic substrate, biofilms, transient storage

Headwater streams are important features in the landscape, where materials from adjacent terrestrial environments are transformed or retained prior to downstream transport (Alexander et al. 2007). Stream biofilms, the complex assemblage of bacteria, fungi, and algae that colonize benthic substrates (Lock et al. 1984), play an essential role in this process, particularly via retention of dissolved nutrients like inorganic N and P (Peterson et al. 2001, Mulholland 2004, Arango et al. 2008). In open-canopy systems where light is abundant, algae are significant biofilm constituents and, hence, autotrophic processes dominate stream metabolism (Minshall et al. 1978, Dodds et al. 2000). In addition to light (Hill 1996), physiochemical characteristics of the aquatic environment, like temperature (Stevenson 1996), nutrient availability (Tank and Dodds 2003, Reisinger et al. 2016), and flow (Biggs et al. 1998, Singer et al. 2010, Haggerty et al. 2014), influence biofilm structure and function. Flow is particularly influential because it varies spatially, creating hydraulic heterogeneity within a stream reach (Biggs et al. 2005), and temporally in response to hydrologic events including storms, snowmelt, and droughts, which can reset algal biofilm colonization by removing biomass (Biggs and Close 1989, Biggs 1995). Moreover, hydrologic extremes are predicted to increase in magnitude and frequency under a changing climate (Uehlinger et al. 2003) with unknown consequences for stream communities.

The temporal sequence of algal colonization and biomass accrual in streams is influenced by similar abiotic factors (Battin et al. 2003, Besemer et al. 2007, Cibils-Martina et al. 2017), with important implications for patterns in autotrophic metabolism that are correlated with nutrient retention (Webster et al. 2003). In particular, assimilatory uptake of both N and P is one mechanism by which algal biofilms regulate nutrient transport to downstream systems (Arango et al. 2008), although dissimilatory (e.g., nitrification, denitrification) and physical processes (sorption) also are important in some streams (Peterson et al. 2001, Mulholland et al. 2008). Benthic substrate provides the habitat for algal colonization (Burkholder 1996), and its characteristics (chemical composition, surface area, and stability) strongly influence benthic biofilms (Besemer 2015). Stable, heterogeneous substrates generally increase algal biomass and productivity

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DOI: 10.1086/699004. Received 19 October 2017; Accepted 19 March 2018; Published online 25 June 2018.
Freshwater Science. 2018. 37(3):000–000.

(Cardinale et al. 2002, Hoellein et al. 2009, Cardinale 2011), particularly while algal biofilms recover after disturbances (Grimm 1987). Therefore, benthic substrate has the potential to influence temporal patterns of biological N and P demand via changes in biomass. Previous studies have linked spatial and temporal variations in nutrient uptake to benthic substrate size (Hoellein et al. 2007) and type (Munn and Meyer 1990, Hoellein et al. 2009), but much of this work has been comparative, and we lack knowledge of the linkages between substrate, biofilms, and nutrient uptake in an experimental context at the reach-scale.

Both biofilm and substrate can influence residence time of water in stream channels by increasing the size of the transient storage zone (Mulholland et al. 1994, Bottacin-Busolin et al. 2009, Orr et al. 2009, Argerich et al. 2011, Aubeneau et al. 2014, 2016). Increased residence times are predicted to influence nutrient uptake by enhancing opportunities for dissolved solutes to interact with stream biofilms (Valett et al. 1996), but multiple investigators have been unable to identify conclusively a relationship between transient storage and nutrient uptake (Triska et al. 1989, Martí et al. 1997, Mulholland et al. 1997, Hall et al. 2002, Bernot et al. 2006). These inconclusive results suggest that variability in the relationship between transient storage and nutrient uptake is mediated by additional controlling variables that may be site-specific, including linkages between biofilm and benthic substrate. For example, transient storage increases as biofilm growth enhances fine-scale, structural complexity in streams (Mulholland et al. 1994, Battin et al. 2003). Alternatively, biofilm accrual can clog interstitial spaces over time (Bottacin-Busolin et al. 2009, Orr et al. 2009, Aubeneau et al. 2016), thereby enhancing accumulation of fine particles (Roche et al. 2017), which ultimately reduces exchange of water between the stream channel and the subsurface (Battin et al. 2003). Thus, the interaction between transient storage and nutrient uptake is complicated by the fact that both metrics covary with factors that are spatially and temporally heterogeneous in the natural environment.

We quantified the influence of benthic substrate size and orientation on nutrient uptake in multiple open-canopy, experimental streams over a temporal sequence of biofilm development at the Notre Dame Linked Experimental Ecosystem Facility (ND-LEEF). Previous research on these experimental streams showed that prior to biofilm growth, substrate composition alone influences how water moves through these systems (Aubeneau et al. 2014) and that biofilm development can alter the signature of substrate on transient storage metrics (Mendoza-Lera and Mutz 2013, Aubeneau et al. 2016). We hypothesized that the influence of substrate on nutrient uptake would be related to substrate size via its influence on surface area (Bott and Kaplan 1985, Mendoza-Lera et al. 2016), and that biofilm colonization and nutrient uptake would be highest in streams dominated by smaller substrates. In addition, we predicted that temporal patterns in uptake would be solute-specific and would vary with biological demand over the trajectory of biofilm colonization, whereas the relationship between nutrient uptake and transient storage would be mediated by both substrate characteristics and biofilm development.

METHODS

Study site

We conducted our study in 4 experimental streams at ND-LEEF (St Joseph County, Indiana). These streams are 50-m-long, concrete-lined systems that receive constant flow (~1.5 L/s) from a groundwater-fed reservoir with very low background nutrients (NH_4^+-N = 5 μg/L, NO_3^--N = 4 μg/L, SRP = 8 μg/L). Stream solute concentrations reflect those of the groundwater aquifer rather than the pervasive eutrophication typically found in the midwestern USA. This feature is unique and advantageous for an experimental facility, where low background nutrient levels facilitate measurements during additions. The 4 streams at ND-LEEF have similar background temperature, conductivity, and pH (Table 1). We manipulated substrate (i.e., size and structure) in each channel (Fig. 1A–D) by lining one with coarse gravel (COBB; median particle size [D_{50}] = 5 cm; Fig. 1A), one with pea gravel (PG; D_{50} = 0.5 cm; Fig. 1B), one with a 50 : 50 mix of pea and coarse gravel (MIX; Fig. 1C), and one with alternating 2-m sections of pea and coarse gravel (ALT; Fig. 1D). Our experiment began in July 2013 when water from the groundwater-fed reservoir was first released into the streams and continued over an ~4-mo colonization period, spanning 115 d. PG and ALT had slightly higher discharge than COBB and MIX (analysis of variance [ANOVA], $p < 0.001$; Tukey Honest Significant Difference [HSD], $p < 0.001$) because of very slight differences in the valves that controlled flow from the reservoir. However, average width and depth did not differ among streams.

Stream characteristics

The streams at ND-LEEF are shallow, open-canopy streams in which assimilatory nutrient uptake is dominated by primary producers. We quantified algal biomass as chlorophyll *a* (Chl *a*) concentration and the accumulation of fine benthic organic matter (FBOM) to represent the mass of both live and dead algae. We collected benthic samples for Chl *a* and FBOM 8 times (days 1, 10, 16, 24, 31, 44, 65, and 115). We inserted an inverted 160-mL specimen container ~2 cm into the stream bed to collect benthic Chl *a* samples of known area at 5 locations that were randomly distributed along each 50-m stream reach (Hoellein et al. 2009). Samples were drained completely, stored on ice, and frozen until analysis. We extracted Chl *a* from each sample and measured it using the cold-methanol fluorometric method (Wetzel and Likens 2001). We also

Table 1. Mean and SE for physical, chemical, and biological characteristics of the 4 experimental streams at the Notre Dame Linked Experimental Ecosystem Facility (ND-LEEF). AFDM = ash-free dry mass. Means with the same superscripts within rows are not significantly different.

	Cobble		Pea gravel		50:50 mixed		Alternating	
Characteristic	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Physical								
Discharge (L/s)	1.56^{A}	0.05	2.04^{B}	0.10	1.62^{A}	0.07	2.02^{A}	0.07
Width (cm)	60.0		56.0		58.9		61.9	
Depth (cm)	6.5		5.1		5.8		5.8	
Temperature (°C)	23.1	1.6	22.8	1.6	22.6	1.7	22.1	1.6
Chemical								
Conductivity (μS/cm)	546.2	8.1	546.4	8.2	544.5	7.5	544.5	7.8
pH	8.6	0.0	8.6	0.0	8.5	0.05	8.5	1.6
NH_4+ (μg/L)	2.8	0.6	3.0	0.5	2.0	0.3	2.8	0.4
NO_3– (μg/L)	3.7	0.7	3.8	0.8	4.0	0.7	4.8	0.8
SRP (μg/L)	3.1	0.8	6.1	0.6	6.4	0.7	6.5	0.8
Biological								
Chlorophyll *a* (mg/m)	12.8	5.1	13.9	3.2	17.5	6.1	12.0	3.6
AFDM (g/cm^2)	0.006^{A}	0.001	0.011^{A}	0.002	0.020^{B}	0.003	0.006^{A}	0.001

collected FBOM samples from 5 randomly distributed locations in each stream. We inserted a 314-cm^2 core (~5-L bottomless bucket) to the bottom of the channel to seal the bottom of the core, vigorously mixed the substrata, and used a 160-mL specimen container to collect a subsample of the homogenized slurry. Within 24 h, we filtered the samples onto a precombusted and preweighed GF/F filter, dried them for 48 h at 60°C, and measured dry mass. We then combusted the filters at 550°C for 1 h, rewet them, and dried them for 48 h at 60°C before measuring the combusted mass. We used the difference between dry and combusted mass as a measure of total FBOM (g ash-free dry mass [AFDM]).

Short-term nutrient additions

To examine the influence of substrate and biofilm accumulation on reach-scale nutrient dynamics, we conducted short-term nutrient additions of NH_4^+, NO_3^-, and PO_4^{3-} in the 4 streams on 8 dates from July 2013 to November 2013 (days 1, 10, 16, 24, 31, 44, 65, and 115 of the colonization sequence). On each date, prior to the start of each nutrient addition, we collected background water-chemistry samples and measured conductivity, temperature, pH, and dissolved O_2 with a Hydrolab Minisonde (Hach, Loveland, Colorado) at stations 10, 20, 30, 40, and 48.5 m downstream of the inlet pipe in each stream. We used stream water to create solute-release solutions for NH_4^+ (as NH_4Cl) and for NO_3^- (as $NaNO_3$) + PO_4^{3-} (SRP as KH_2PO_4), with 300 g of NaCl added to each solution to serve as a conservative tracer. For each addition, we used a peristaltic pump to drip the release solution into the stream at a constant rate of 20 mL/min until stream concentrations reached a plateau (~45 min) identified by monitoring conductivity at the bottom of each reach. For each addition, we increased nutrients ~25, 50, and 20 μg/L above background concentrations for NH_4^+, NO_3^-, and SRP, respectively. At plateau, we collected and filtered 3 replicate water samples at each sampling station, placed them on ice, and transported them to the laboratory where they were frozen until later analysis. We quantified NH_4^+ with the phenol-hypochlorite method (Solorzano 1969), NO_3^- with the Cd-reduction method (APHA 2012), and SRP with the ascorbic acid method (Murphy and Riley 1962) on a Lachat Flow Injection Autoanalyzer (Lachat Instruments, Loveland, Colorado).

We calculated nutrient uptake length (S_w) of each solute on each sampling date by dividing the background-corrected nutrient concentration by the background-corrected conductivity and plotted the natural logarithm of this ratio against distance downstream. This approach accounts for dilution, but dilution is minimal in the concrete-lined, experimental channels at ND-LEEF. The slope of the regression line is the longitudinal uptake rate (k) and the inverse of k is S_w (m; Stream Solute Workshop 1990). Over the experiment, we measured 92 longitudinal uptake rates (k): 32 for NH_4^+ and SRP (4 streams × 8 dates), and 28 for NO_3^- (4 streams × 7 dates). In general, longitudinal uptake measurements were robust. R^2 values for regressions on the slope (k) of dilution-corrected concentration vs distance ranged from 0.88 to 0.99 for NH_4^+ (mean = 0.96 ± 0.01), 0.58 to 0.99 for NO_3^- (mean = 0.87 ± 0.02), and 0.74 to 0.99 for SRP (mean = 0.92 ± 0.01) and were statistically significant

Figure 1. Substrate configuration before and after (inset) biofilm colonization in cobble (COBB) (A), pea gravel (PG) (B), 50:50 mixed cobble and pea gravel (MIX) (C), and alternating cobble and pea gravel (ALT) (D). Each stream reach received regulated flow (discharge = 1.5 L/s) from a low-nutrient groundwater reservoir, but biofilm development was visually different in each system.

($p < 0.05$). Only 1 regression was not significant (PG on day 16; $R^2 = 0.17$, $p > 0.05$ for NO_3^-), and we excluded the resulting k for this relationship from further analyses. Despite the highly controlled nature of the experimental streams at ND-LEEF, small differences in discharge did arise among the experimental streams, so we also calculated uptake velocity (v_f) as discharge/width/S_w to compare nutrient demand within and among streams through time (Stream Solute Workshop 1990, Davis and Minshall 1999, Hoellein et al. 2007). We calculated areal uptake rate (U) by multiplying v_f by background nutrient concentration, which we then divided by Chl *a* and AFDM (mg/m^2) to calculate areal uptake per biological unit (e.g., U_{chla} = mg NH_4^+-N mg^{-1} Chl *a* d^{-1}).

Transient storage metrics

We conducted additions of the conservative tracer Rhodamine WT (RWT) on 5 separate sampling dates to examine changes in transient storage over the trajectory of bio-

film growth with methods described by Aubeneau et al. (2014, 2016). We released a pulse of RWT at the top of each stream and documented the breakthrough curve (BTC) at the bottom of each stream (48.5 m downstream) with a Hydrolab MS5 Minisonde (Hach). We analyzed each BTC with a continuous-time random walk (CTRW) transport model (Berkowitz et al. 2006, Aubeneau et al. 2015), which is similar to transient storage models used in previous studies (e.g., Bencala and Walters 1983) where results were modeled for an exponential residence time in 1 immobile zone (Aubeneau et al. 2016). From the best-fit model, we estimated the parameters, velocity (v, m/s), dispersion (m^2/s), and exchange rate of water between the main channel and transient storage (k_1, 1/s), which were previously compared among the streams at ND-LEEF by Aubeneau et al. (2016). In these systems, the exchange represented by k_1 is constrained to the 'micro' hyporheic zone (cm-scale) because the streambeds at ND-LEEF are lined with concrete, which limits exchange with lateral and deep subsurface hyporheic zones (m-scale). We also calculated the exchange rate of water between transient storage zones and the main channel (expressed as k_1/k_2), which is a ratio similar to the relative size of the transient storage zone (i.e., A_s/A; Bencala and Walters 1983), for comparison to previous studies.

Statistical analyses

We compared k among substrate treatments on each sampling date with analysis of covariance (ANCOVA), where a significant interaction term in the ANCOVA model denotes a significant difference among substrate treatments. We then used repeated-measures analysis of variance (rmANOVA) to test for differences in biological characteristics or nutrient uptake metrics, including S_w, v_f, and U, among substrate treatments. We also compared biological characteristics among streams on each date with 1-way ANOVA with a Bonferroni adjusted p-value to test for significance given 8 sampling dates ($p = 0.05/8 = 0.00625$). Last, we used Pearson's correlation to examine the relationship between functional metrics (i.e., nutrient uptake, transient storage) and biological characteristics. All data were examined for normality with the aid of residual plots and the Shapiro–Wilk test ($p > 0.05$), and in the case of rmANOVA, sphericity with the Mauchly test ($p > 0.05$), followed by either $\log(x)$- or $\sqrt{(x)}$-transformation when necessary. All data analyses were performed in R (version 3.3.1; R Project for Statistical Computing, Vienna, Austria).

RESULTS

Biofilm characteristics

Biofilm Chl *a* generally increased over time, from 0.4 to 2.8 μg/cm^2 in ALT, 0.5 to 4.6 μg/cm^2 in COBB, 0.7 to 4.8 μg/cm^2 in MIX, and 0.4 to 3.0 μg/cm^2 in PG (Fig. 2A). Chl *a* peaked in ALT and PG on day 65, and in COBB and MIX on day 115. Chl *a* was ~4× higher in COBB and MIX than in PG on the last day of the study (1-way ANOVA, $F_{3,16} = 11.7$, $p < 0.001$; Tukey HSD, $p < 0.001$), but because of variation over time and space, overall, Chl *a* did not differ among substrate treatments (rmANOVA, $p > 0.05$). FBOM increased from 0.002 to 0.009 g AFDM/cm^2 in ALT, 0.002 to 0.013 g AFDM/cm^2 in COBB, 0.009 to 0.029 g AFDM/cm^2 in MIX, and 0.003 to 0.019 g AFDM/cm^2 in PG (Fig. 2B). In contrast to Chl *a*, FBOM peaked on day 31 in MIX, day 65 in PG, and day 115 in ALT and COBB. On most dates, FBOM differed among the 4 substrate treatments (1-way ANOVA, $p < 0.00625$ for all; Fig. 2B). MIX had significantly more FBOM than ALT and COBB at the start of the experiment (Tukey HSD, $p < 0.001$) and this difference persisted until day 65 when FBOM was 3× higher in PG than COBB (Tukey HSD, $p = 0.005$), but no other differences were significant. Overall, FBOM was higher in MIX than in the other 3 substrate treatments (rmANOVA, $p < 0.001$; Tukey HSD, $p < 0.001$ for all).

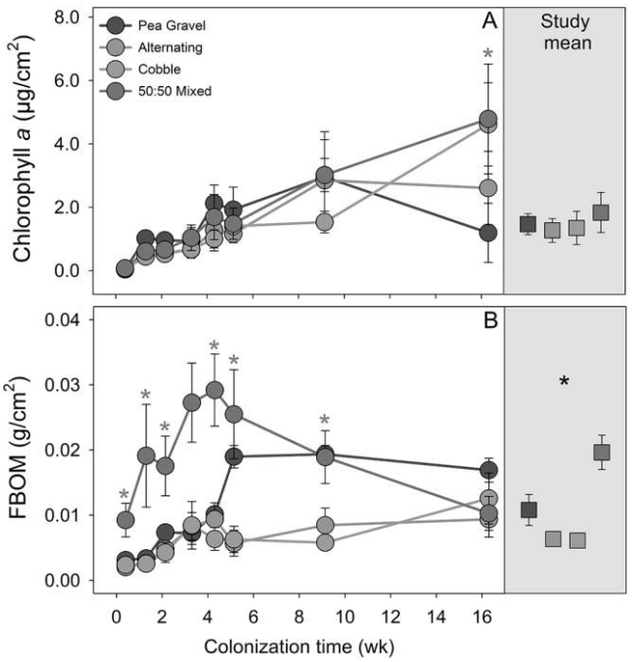

Figure 2. Mean (±SE) chlorophyll *a* (A) and fine benthic organic matter (FBOM) (B) in each stream on days 1, 10, 16, 24, 31, 44, 65, and 115 of biofilm colonization or development. Asterisks denote significant differences among streams on each sampling date (1-way analysis of variance [ANOVA], $p < 0.00625$). Stream means are shown in gray boxes, and black asterisks denote significant differences among streams (repeated measures ANOVA, $p < 0.05$).

Does substrate influence nutrient uptake metrics?

For each of the 92 releases on 8 sampling dates, we report nutrient removal as the longitudinal uptake rate (k),

the R^2 of the regression, and the 95% confidence interval for k for each solute (Table S1). In general, substrate influenced k during later dates, but the effect of substrate varied among solutes. For NH_4^+, substrate consistently influenced k after the first 3 sampling dates (ANCOVA, distance × stream, $p < 0.05$ for days 24–115). In contrast, for NO_3^-, the effect of substrate on k was variable through time but significant on 5 of the 8 sampling dates (ANCOVA, distance × stream, $p \leq 0.05$ for days 10, 24, 31, 65, and 115). For SRP, the effect of substrate on k was similarly variable, and k differed among substrate treatments on days 10, 31, 65, and 115 (ANCOVA, distance × stream, $p < 0.05$ for all).

S_w did not differ among substrate treatments over time for any solute (rmANOVA, $p > 0.05$; Fig. 3A, C, E). Across all sampling dates, S_w ranged from 8.3 to 64.7 m for NH_4^+ (Fig. 3A), 31.8 to 295.6 m for NO_3^- (Fig. 3C), and 24.4 to 208.2 m for SRP (Fig. 3E). Average S_w was generally longest in COBB for NH_4^+ (mean = 43.1 ± 4.8 m), NO_3^-(185.9 ± 36.2 m), and SRP (mean = 65.9 ± 20.8 m). S_w for each solute did not differ significantly among sampling dates (rmANOVA, $p > 0.05$).

The overall pattern in nutrient demand (v_f) for each solute varied among substrate treatments over the trajectory of biofilm development (Fig. 3B, D, F). Averaged across all sampling dates, v_f in the 4 substrate treatments ranged from 4.0 to 8.5 mm/min for NH_4^+, 1.2 to 2.0 mm/min for NO_3^-, and 3.1 to 5.4 mm/min for SRP. v_f for SRP differed significantly among substrate treatments (rmANOVA, $p < 0.001$; Fig. 3F) and was ~1.5× faster in PG than in all other streams (Tukey HSD, $p < 0.01$ for all). v_f did not differ among substrate treatments for either N solute, but mean v_f was highest in PG for NH_4^+ (8.4 mm/min; Fig. 3B) and NO_3^- (2.0 mm/min; Fig. 3D) on day 65. v_f varied through time (rmANOVA, $p < 0.001$) only for SRP and was higher on days 10 and 115 than on all other sampling dates (Tukey HSD, $p < 0.05$ for all) except day 65, which was intermediate (Fig. 3F).

Across all sampling dates, U ranged from 15.2 to 31.2 mg NH_4^+-N m^{-2} d^{-1} (Fig. 4A), 5.0 to 10.9 mg NO_3^--N m^{-2} d^{-1} (Fig. 4D), and 29.1 to 48.3 mg SRP m^{-2} d^{-1} (Fig. 4G). Substrate influenced U for both NH_4^+ (rmANOVA, $p = 0.03$; Fig. 4A) and SRP (rmANOVA, $p = 0.01$; Fig. 4G). U for both NH_4^+ and

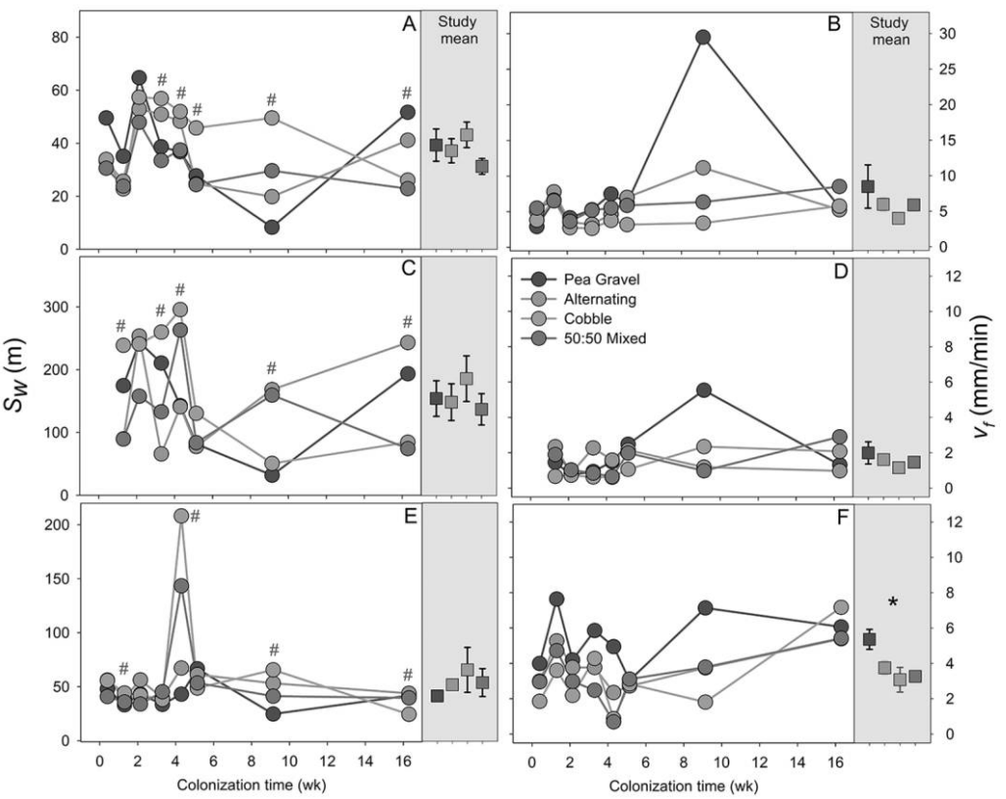

Figure 3. Uptake length (S_w) (A, C, E) and uptake velocity (v_f) (B, D, F) for NH_4^+ (A, B), NO_3^- (C, D), and soluble reactive P (SRP) (E, F) on days 1, 10, 16, 24, 31, 44, 65, and 115 of biofilm colonization or development. The longitudinal uptake rate (k), or slope of the regression relationship between distance and background-corrected nutrient concentration, was significantly different among substrate treatments on sampling dates denoted with gray pound signs. Stream means are shown in gray boxes, and black asterisks denote significant differences among streams (repeated measures analysis of variance, $p < 0.05$).

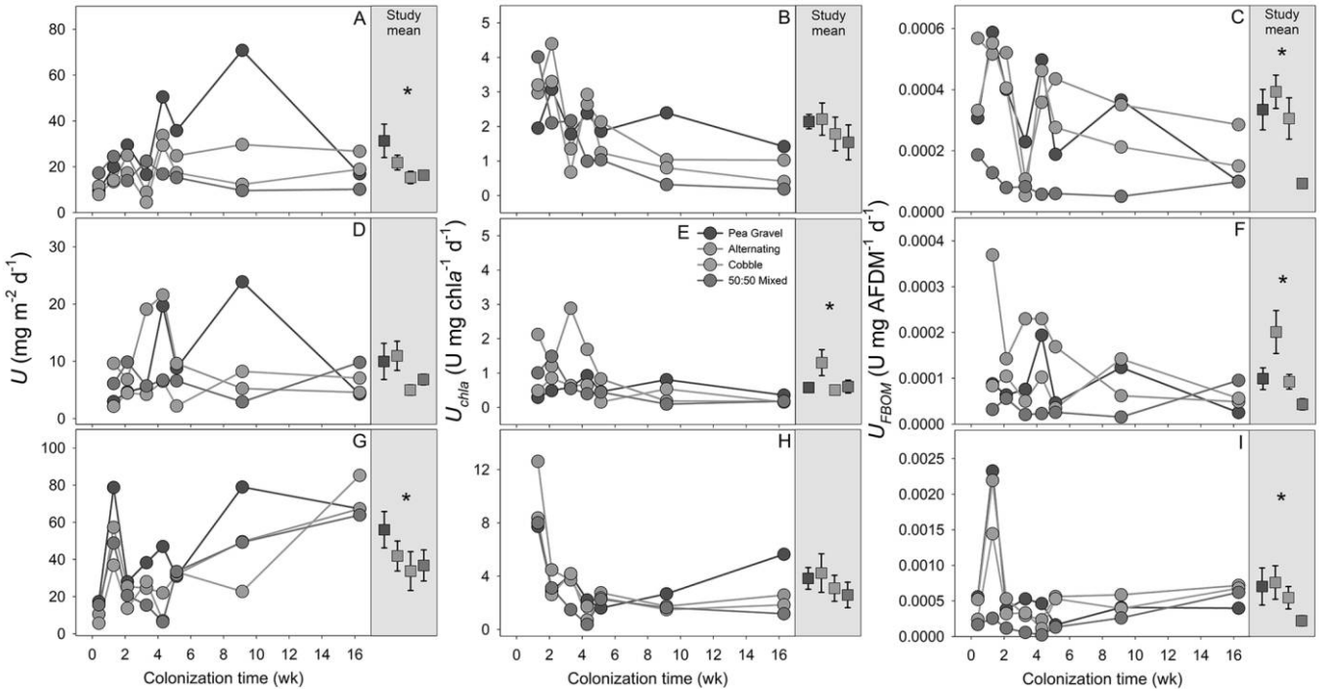

Figure 4. Areal uptake rate (U) (A, D, G), U/mg chlorophyll a (U_{chla}) (B, E, H), and U/g fine benthic organic matter (U_{FBOM}) (C, F, I) for NH_4^+ (A–C), NO_3^- (D–F), and soluble reactive P (SRP) (G–I) on days 1, 10, 16, 24, 31, 44, 65, and 115 of biofilm colonization or development. Stream means are shown in gray boxes, and black asterisks denote significant differences among streams (repeated measures analysis of variance, $p < 0.05$). AFDM = ash-free dry mass.

SRP was 1.5 to 2× higher in PG than in COBB or MIX (Tukey HSD, $p < 0.05$ for all). U differed among sampling dates only for SRP (rmANOVA, $p < 0.001$) and was higher on day 115 than all other days except days 10 and 65 (Tukey HSD, $p < 0.05$ for all).

After day 1, as biofilm Chl a began accumulating in each stream, U_{chla} ranged from 1.5 to 2.2 mg NH_4^+-N mg^{-1} Chl a d^{-1} (Fig. 4B), 0.5 to 1.3 mg NO_3^--N mg^{-1} Chl a d^{-1} (Fig. 4E), and 2.6 to 3.8 mg SRP mg^{-1} Chl a d^{-1} (Fig. 4H). Substrate influenced U_{chla} only for NO_3^- (rmANOVA, $p = 0.03$; Fig. 4E). U_{chla} was higher in ALT than in COBB (Tukey HSD, $p = 0.01$). U_{chla} did not differ among sampling dates for any solute (rmANOVA, $p > 0.05$), a result suggesting that uptake per unit chl a biomass was relatively consistent through time.

FBOM was present from the start of the experiment (Fig. 2B), and U_{AFDM} ranged from 0.0001 to 0.0004 mg NH_4^+-N mg^{-1} AFDM d^{-1} (Fig. 4C), 0.00003 to 0.0002 mg NO_3^--N mg^{-1} AFDM d^{-1} (Fig. 4F), and 0.0002 to 0.0007 mg SRP mg^{-1} AFDM d^{-1} (Fig. 4I). U_{AFDM} for all 3 solutes differed among substrate treatments (rm ANOVA, $p < 0.05$ for all). U_{AFDM} for NH_4^+ was lower in MIX than in all other streams (Tukey HSD, $p < 0.001$; Fig. 4C), U_{AFDM} for NO_3^- was higher in ALT in than COBB and MIX (Tukey HSD, $p < 0.01$; Fig. 4F), U_{AFDM} for SRP was lower in MIX than in ALT and PG (Tukey HSD, $p < 0.05$; Fig. 4I). U_{AFDM} for NH_4^+ differed among sampling dates (rmANOVA, $p = 0.002$) and was lower on day 24 than on all other sampling dates (Tukey HSD, $p < 0.05$ for days 1, 10, 16, and 31; Fig. 4C). U_{AFDM} for SRP also differed among sampling dates (rmANOVA, $p < 0.001$) and was higher on day 10 than on all other days (Tukey HSD, $p = 0.05$ for all; Fig. 4I).

What were the major drivers of nutrient uptake?

Across substrate treatments and solutes, algal biomass was the major control on nutrient uptake. k_1 in our study streams varied from 0.062 to 0.074/min in PG, 0.057 to 0.091/min in ALT, 0.073 to 0.096/min in COBB, and 0.065 to 0.082/min in MIX. In each stream, k_1 was highest on day 1 and decreased over time. k_2 also decreased in each stream over time. Therefore, we examined the correlations of k_1 and k_2 with biological characteristics. k_1 and k_2 were negatively correlated with chl a (Pearson, k_1: $r = -0.53$, $p = 0.03$; k_2: $r = -0.54$, $p = 0.02$; Fig. 5A and data not shown, respectively) and FBOM (Pearson, k_1: $r = -0.57$, $p = 0.01$; k_2: $r = -0.47$, $p = 0.05$; data not shown, respectively). SRP v_f and U were negatively correlated with k_1 (Pearson, $r = -0.51$ and -0.67, respectively, $p < 0.05$ for both) and k_2 (Pearson, $r = -0.46$ and -0.61, respectively, $p < 0.05$ for both). Neither k_1 nor k_2 were significantly cor-

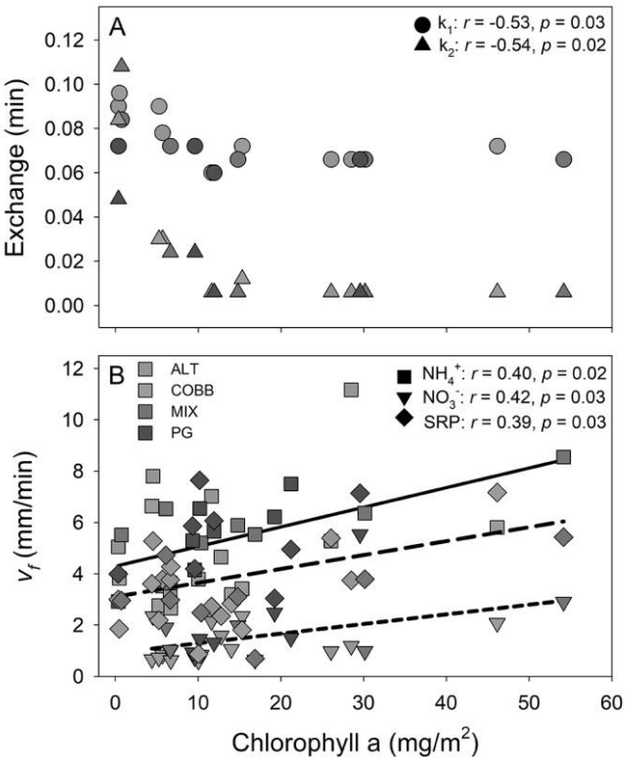

Figure 5. Correlations for exchange of water from the main channel to the subsurface (k_1) and from the subsurface to the main channel (k_2) (A), and nutrient demand (v_f) for NH_4^+, NO_3^-, and soluble reactive P (SRP) (B) with chlorophyll *a* (Chl *a*). Lines are included in panel B to illustrate the differences among solute types.

related with uptake metrics for NH_4^+ or NO_3^- (Pearson, $p > 0.05$), but trends indicated similarly inverse relationships in which N uptake increased as k_1 and k_2 decreased.

Metrics for NH_4^+ were correlated with biofilm Chl *a* across substrate treatments, suggesting that as Chl *a* increased, S_w decreased (Pearson; $r = -0.42$, $p = 0.02$; Table 2) and v_f increased (Pearson, $r = 0.40$, $p = 0.02$; Fig. 5B, Table 2). For NO_3^-, only v_f was positively correlated with chl *a* (Pearson, $r = 0.42$, $p = 0.03$; Fig. 5B, Table 2). For SRP, both v_f and U were positively correlated with Chl *a* (Pearson, $r = 0.39$ and 0.63, respectively, $p < 0.05$ for both; Fig. 5B, Table 2).

Within individual streams, the effect of algal biomass on nutrient uptake varied among substrate treatments and with uptake metrics. In PG, for both NH_4^+ and NO_3^-, S_w of both N solutes was negatively correlated with Chl *a* (Pearson, NH_4^+: $r = -0.76$, $p = 0.03$; NO_3^-: $r = -0.92$, $p < 0.01$), whereas v_f and U were both positively correlated with Chl *a* (Pearson, $r \geq 0.80$, $p < 0.05$ for all). In MIX, only v_f for NH_4^+ was correlated with Chl *a* (Pearson, $r = 0.79$, $p = 0.02$). In COBB, v_f and U for SRP were positively correlated with Chl *a* (Pearson, $r = 0.75$ and 0.89, respectively, $p < 0.05$ for both). In MIX, U for SRP was positively correlated with Chl *a* (Pearson, $r = 0.70$, $p = 0.05$). In contrast, the only uptake metric correlated with FBOM (representative of both living and dead organic matter) was v_f for SRP in COBB (Pearson, $r = 0.73$, $p = 0.04$; Table 2).

DISCUSSION

Agricultural and urban land use systematically reduce habitat heterogeneity and complexity in streams (Allan 2004). The substratum of these simplified systems is often homogeneous and dominated by fine sediments that are unstable at high flows, resulting in disturbance of biological assemblages and resultant ecosystem processes (O'Connor et al. 2012). In addition, streams draining agricultural and urban land are often channelized and lack geomorphological complexity, which reduces water residence times and constrains nutrient removal (e.g., Gooseff et al. 2007, Sheibley et al. 2014). Enhancing habitat heterogeneity and complexity is often a key goal of stream restoration, but most investigators quantify the effects of reintroducing large-scale, geomorphic features (i.e., pools and riffles; Bukaveckas 2007) or re-establishing floodplain connectivity (Kaushal et al. 2008, Roley et al. 2012, McMillan and Noe 2017). Our study provided a unique opportunity to isolate the influence of benthic substrate on nutrient removal, which can be challenging given that human-induced changes to physical and chemical characteristics of stream ecosystems often covary. Overall, we found that substrate-specific biofilm growth influenced nutrient processing. This result could have important implications for management and restoration of streams to optimize ecosystem function.

Numerous studies have documented the influence of physical, chemical, and biological characteristics on nutrient dynamics, including transient storage (Grimm and Fisher 1984, Jones and Holmes 1996, Valett et al. 1996), substrate (Munn and Meyer 1990, Hoellein et al. 2007), and algal biomass (Martí et al. 1997). Nevertheless, the interactions among these factors and their subsequent influence on nutrient uptake are largely unexplored because few investigators have experimentally manipulated substrate at the reach-scale and examined its influence on nutrient dynamics (but see Battin et al. 2003, Orr et al. 2009). In our study, substrate manipulation in experimental streams spanned a range of sediment size-classes from fine to very coarse gravel under both homo- and heterogeneous conditions. Previous studies of the streams at ND-LEEF demonstrated that uncolonized benthic substrate composition influences how water moves into and out of the hyporheic zone (Aubeneau et al. 2014) and that subsequent biofilm development alters the signature of transient storage (Aubeneau et al. 2016).

We built on this previous work and examined a biofilm colonization sequence. We found that longitudinal nutrient removal (k) varied among substrate treatments on most sampling dates, particularly after biofilm development.

Table 2. Pearson's correlation statistics for correlations between each nutrient uptake metric (S_w, v_f, and U) for NH_4^+, NO_3^-, and soluble reactive P (SRP) and in-stream characteristics, including chlorophyll *a* (Chl *a*) and fine benthic organic matter (FBOM). Correlations were examined across all streams (All Streams) and within individual streams. COBB = cobble, PG = pea gravel, MIX = 50 : 50 mixed cobble and pea gravel, ALT = alternating cobble and pea gravel.

		NH_4^+						NO_3^-						SRP					
		S_w (m)		v_f (mm/min)		U (mg m^{-2} d^{-1})		S_w (m)		v_f (mm/min)		U (mg m^{-2} d^{-1})		S_w (m)		v_f (mm/min)		U (mg m^{-2} d^{-1})	
		r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Chl *a*	All streams	**−0.42**	**0.02**	**0.40**	**0.02**	0.26	0.15	−0.35	0.07	**0.42**	**0.03**	0.14	0.47	−0.10	0.58	**0.39**	**0.03**	**0.63**	**0.00**
	PG	**−0.76**	**0.03**	**0.80**	**0.02**	**0.94**	**0.00**	**−0.92**	**0.00**	**0.89**	**0.01**	**0.94**	**0.00**	−0.18	0.67	0.24	0.57	0.52	0.19
	ALT	−0.28	0.50	0.54	0.16	0.67	0.07	0.42	0.35	−0.55	0.20	−0.46	0.30	−0.01	0.98	0.24	0.56	0.65	0.08
	COBB	−0.35	0.39	0.38	0.35	0.30	0.47	−0.65	0.12	0.71	0.08	0.49	0.26	−0.20	0.64	**0.75**	**0.03**	**0.89**	**0.00**
	MIX	−0.40	0.32	**0.79**	**0.02**	−0.68	0.06	−0.24	0.61	0.59	0.16	0.18	0.71	−0.01	0.99	0.49	0.22	**0.70**	**0.05**
FBOM	All streams	−0.28	0.12	0.25	0.17	0.22	0.23	−0.20	0.31	0.14	0.47	0.08	0.69	0.09	0.64	−0.07	0.70	0.07	0.71
	PG	−0.52	0.19	0.56	0.15	0.60	0.12	−0.71	0.08	0.66	0.11	0.47	0.28	0.18	0.67	−0.05	0.91	0.31	0.45
	ALT	0.29	0.49	0.00	1.00	0.58	0.13	0.20	0.66	−0.36	0.43	0.27	0.56	0.07	0.86	−0.05	0.91	0.26	0.53
	COBB	0.01	0.99	0.06	0.88	0.18	0.68	−0.38	0.40	0.47	0.29	0.47	0.29	−0.11	0.80	**0.73**	**0.04**	0.75	0.03
	MIX	0.18	0.68	−0.36	0.39	0.36	0.38	0.54	0.21	−0.68	0.09	−0.44	0.33	0.58	0.13	−0.66	0.08	−0.46	0.25

Longitudinal nutrient removal was generally lowest in COBB, making S_w generally longest in COBB. Patterns in nutrient demand relative to concentration (v_f), which accounted for small variations in stream discharge over time and among streams (Stream Solute Workshop 1990), also varied among substrate treatments. v_f of all 3 solutes was 1.2 to 1.5× higher in PG than all other streams. U was also highest in PG, although areal uptake expressed per unit biomass (U_{chla} and U_{AFDM}) emphasized the role of biotic control on nutrient uptake by damping substrate-specific variations. Overall, our results indicate that benthic substrate alters nutrient cycling in streams through its influence on biofilm development.

Solute-specific trends were evident across uptake metrics

Trends were similar, but the magnitude of individual uptake metrics varied with solute. For example, v_f of all 3 solutes fell within the range reported in a previous meta-analysis (Ensign and Doyle 2006). v_f was highest for NH_4^+ (range = 2.5–17.0 mm/min), followed by SRP (range = 1.5–6.6 mm/min) and NO_3^- (range = 0.8–4.2 mm/min), demonstrating that trends in these experimental streams reflected patterns found in natural streams. Similarly, average $NH_4^+ v_f$ was highest (6.1 mm/min) followed by SRP (3.9 mm/min) and NO_3^- (1.7 mm/min), making our results consistent with those of previous studies in which demand for NH_4^+ was relatively higher than for SRP and NO_3^- when spatial and temporal patterns of nutrient uptake dynamics were examined (Simon et al. 2005, Martí et al. 2009).

Stream biofilm growth and productivity commonly are limited by the availability of inorganic N, P, or a combination of both (Francoeur 2001, Tank and Dodds 2003). Low background N and P concentrations (<10 μg/L for each) at ND-LEEF resulted in very low N : P ratios (<2) and suggested that biofilms in these systems probably were N-limited (Grimm and Fischer 1986, Grimm 1987), resulting in higher inorganic N demand (higher v_f) relative to inorganic P. N demand is commonly high in open-canopy systems dominated by algal biofilms (Grimm 1987, Dodds et al. 2000), and nutrient demand (as v_f) for both NH_4^+ and NO_3^- was higher in streams at ND-LEEF than in a similar-sized prairie stream (NH_4^+: 0.27–2.65 mm/min, NO_3^-: 0.4–0.7 mm/min; Dodds et al. 2002). Results at ND-LEEF also indicate preferential demand for the energetically favorable NH_4^+ over NO_3^- as an inorganic N source. This preference has been shown previously for individual streams (e.g., Mulholland et al. 2000, Day and Hall 2017) and streams spanning a range of biomes (Webster et al. 2003) and sizes (Hall et al. 2013).

Removal of inorganic N from the water column can occur via assimilatory and dissimilatory (i.e., nitrification, denitrification) pathways. Ribot et al. (2017) found in Mediterranean streams that assimilatory uptake and nitrification contributed equally to NH_4^+ uptake (expressed as U), whereas assimilation was the dominant pathway for NO_3^- uptake. We did not observe increases in NO_3^- concentration during NH_4^+ releases. This result suggests that assimilation by algal biofilms was the main mechanism of inorganic N removal from the water column. We assume that denitrification was low in the aerobic conditions of our streams, but anoxic microsites might have been present (Holmes et al. 1996). In contrast to N, inorganic P uptake can be influenced by abiotic sorption. U for SRP (mean U = 36.3 mg m^{-2} d^{-1}) was considerably higher than for NH_4^+ (mean U = 21.1 mg m^{-2} d^{-1}) and NO_3^- (mean U = 8.2 mg m^{-2} d^{-1}), and may suggest that a portion of SRP removal from the water column included abiotic sorption, which we did not quantify directly. However, previous investigators have reported mixed results on the contribution of abiotic sorption to overall P removal rates (Mulholland et al. 1983, Aldridge et al. 2010, Price and Carrick 2013), and higher U for SRP may reflect the role of heterotrophic microbes associated with decomposing organic matter, including algal senescence (Allan 1995, Mulholland 1996, Rier and Stevenson 2001).

Substrate treatment influenced colonizable surface area in streams

Despite variation among solutes, benthic substrate composition and orientation influenced biofilm development, which controlled overall patterns of nutrient removal (as k) and demand (as v_f) among the 4 streams at ND-LEEF. Benthic substrate provides the habitat template for biofilm development in streams (Burkholder 1996), and biofilm structure and function can vary with stability, size, heterogeneity, chemical composition, and roughness of benthic substrate (Cardinale et al. 2002, Hoellein et al. 2007, Bergey et al. 2010, Besemer 2015). The streams at ND-LEEF were lined with rocks of similar geologic origin. Therefore, substrate size and heterogeneity were the most likely explanations for variability in nutrient removal and demand among the 4 streams. However, physical features can influence biological processes only indirectly, and we suggest that the effect of substrate was largely caused by differences in the surface area available for algal biofilm colonization. Substrate size defines surface area, thereby determining the physical habitat available for colonization by biological communities (Hargrave 1972, Bott and Kaplan 1985, Marxsen and Witzel 1990). Larger substrates have lower surface-to-volume ratios, which means that 1 m^2 of benthic area will have less colonizable surface area than the same area with smaller substrates. For the streams at ND-LEEF, substrate surface area (per cm^2 of streambed) was lowest in COBB (19 cm^2), followed by MIX (26 cm^2) and ALT (41 cm^2), and highest in PG (70 cm^2), which is consistent with an inverse relationship between grain size and surface area (Bott and Kaplan

1985, Mendoza-Lera et al. 2016) and probably contributes to lower removal rates (k) and relative nutrient demand (as v_f) in COBB for all 3 solutes.

In natural streams, a trade-off often exists between increased surface area and algal colonization because smaller substrata are unstable and vulnerable to recurring flow disturbances, resulting in lower biofilm biomass than might be predicted based on surface area alone (Romani and Sabater 2001, Hoellein et al. 2009). Investigators examining the role of substrate size on biofilm development generally have emphasized the importance of large, stable substrata for algal biomass accumulation, particularly when considering the effect of disturbance (Fisher et al. 1982, Uehlinger 1991). Others have associated higher nutrient removal (i.e., shorter S_w) with biological assemblages growing on large, stable substrata in reaches dominated by cobble and bedrock compared to reaches dominated by sand and small gravel (Munn and Meyer 1990, Martí and Sabater 1996). Nevertheless, accumulation of algal biomass on small substrata can be substantial in some systems during periods of prolonged baseflow (Tett et al. 1978) or dry seasons (Townsend and Padovan 2005). Our study demonstrates that the interaction of substrate size and algal biofilm development has the potential to enhance nutrient removal, but the effect depends on flow conditions because of the variable influence of disturbance (Fisher and Grimm 1988, Luce et al. 2010).

The influence of substrate was strongly mediated by biofilm

Nutrient demand was positively correlated with Chl *a* across substrate types and solutes, suggesting that algal biomass controlled biological demand for inorganic N and P. The ample light, predominance of inorganic substrata, and steady flows across streams at ND-LEEF were ideal for algal growth (Boston and Hill 1991, Hill et al. 1995, Besemer et al. 2007). Some investigators have found that inorganic N demand increases with Chl *a* (Niyogi et al. 2004), whereas others have reported only weak relationships (e.g., Simon et al. 2005), possibly as a result of changing flows (Biggs and Close 1989, Biggs 1995), light availability (Hill 1996), and grazing activity (Rosemond et al. 1993) that result in spatial and temporal variability in algal biomass. Nevertheless, algal constituents of epilithic biofilms can control uptake of NH_4^+ and NO_3^- (Davis and Minshall 1999, Kemp and Dodds 2002), and N demand is related to functional metrics like autotrophic assimilation and rates of primary production (Peterson et al. 2001, Hall and Tank 2003, Webster et al. 2003, Garcia et al. 2016).

Algal biomass was correlated with SRP v_f at ND-LEEF, consistent with previous studies in which epilithic biomass explained variation in SRP demand (Martí et al. 2009). Both N and P are needed to sustain autotrophic metabolism (Bothwell 1989, Francoeur 2001), and in an interbiome comparison, Mulholland et al. (2001) found that water-column SRP concentration was a significant predictor of stream gross primary production. However, unlike NH_4^+ and NO_3^- v_f, which were similar among substrate types, SRP demand was significantly higher in PG than in other streams, perhaps reflecting additional demand for SRP by heterotrophic assemblages associated with decomposing senesced algae, which accumulated in the increased interstitial spaces of PG. Previous investigators have shown that heterotrophic microbes associated with photoautotrophs contribute to nutrient uptake in streams (Allan 1995, Mulholland 1996).

We expressed U in 3 different ways: per unit streambed area (U), per unit algal biomass (U_{chla}), and per unit benthic organic matter combining live and dead biomass (U_{AFDM}). Similar to v_f, U was generally highest in PG reflecting higher surface area for biofilm colonization resulting from the smaller substrate size. In other studies of substrate-specific nutrient uptake, uptake rates varied among substrate types (Kemp and Dodds 2002, Hoellein et al. 2009), but in most of these studies, individual substrata were isolated in chamber incubations. Fewer investigators have directly measured the effect of substrate at the reach-scale (but see Martí and Sabater 1996). Our results are similar to those of Munn and Meyer (1990) who found that areal uptake of NO_3^- was nearly 13× higher in a gravel than in a cobble reach. Expressing U per unit Chl *a* normalized the differences among streams and showed that among-stream variation in areal uptake was strongly controlled by algal biomass at ND-LEEF. This result is consistent with results of previous work in open-canopy systems that suggested algal biomass increases the effective surface area of the streambed (Dodds et al. 2004). In contrast, expressing U per unit benthic organic matter revealed that uptake of inorganic N and P was higher in ALT than the other 3 streams, which had relatively low FBOM accumulation. Substrate heterogeneity can stimulate rates of primary production without altering total biomass, suggesting a change in biofilm efficiency (Cardinale et al. 2002) that may partially explain the higher U_{AFDM} in ALT that we observed across solutes. In sum, comparing U expressed per unit streambed vs algal and organic matter mass indicated a synergistic effect of substrate surface area and biofilm colonization on nutrient processing in streams at ND-LEEF.

Substrate and biofilm development interacted to influence transient storage

Accumulation of biofilm biomass throughout the colonization sequence at ND-LEEF influenced transient storage by limiting the exchange of surface flows with subsurface, and ultimately influencing the contribution of subsurface

processes to nutrient uptake. We found that k_1 was negatively correlated with Chl *a*, indicating that exchange between the water column and subsurface transient zones decreased as algal biomass increased. The experimental streams at ND-LEEF are underlain with concrete. Therefore, surface–subsurface interactions at the sediment–water interface are limited to the microhyporheic scale (Shogren et al. 2017). Nevertheless, patterns in k_1 reflected changes in the amount of water and dissolved solutes entering the streambed as biofilm growth and FBOM accumulation clogged interstitial spaces. Various investigators have demonstrated that biofilm development (Mulholland et al. 1994, Battin et al. 2003) and substrate structure (Argerich et al. 2011, Aubeneau et al. 2014) can enhance fine-scale complexity and increase the influence of transient storage in streams. However, investigators working in experimental flumes also found that biofilm growth can reduce subsurface exchange over time (Battin et al. 2003, Bottacin-Busolin et al. 2009, Orr et al. 2009), particularly under conditions of controlled flow and constrained hyporheic zones like those in the streams at ND-LEEF. If subsurface processes were controlling nutrient uptake at ND-LEEF, we would expect a similar, inverse relationship between nutrient uptake metrics and algal biomass. Instead, we found that N and P demand (as v_f) increased with algal biomass despite decreasing hyporheic exchange, providing further evidence that assimilatory uptake by algal biofilms on substrate surfaces dominated nutrient dynamics in our study streams.

The theoretical basis for the relationship between transient storage and nutrient uptake metrics is based on the idea that water retention, particularly in slow-moving zones in the streambed or within subsurface sediments, should increase interaction time between dissolved nutrients and biota (Valett et al. 1996). Therefore, transient storage zones are predicted to influence nutrient uptake at the reach-scale, and many investigators have focused on quantifying this relationship based on the relative size of the transient storage zone (i.e., A_s/A: Valett et al. 1996, Bernot et al. 2006; or k_1/k_2: Hall et al. 2002). The range of k_1/k_2 values measured in the ND-LEEF streams (0.5–8.6) was similar to those reported previously for headwater streams (0.1–5, Valett et al. 1996; 1–18, Martí et al. 1997; 0.6–0.71, Hall et al. 2002). However, focusing on this metric alone could have been misleading because k_2, the exchange of water from transient storage back to the main channel also was influenced by algal biomass. When relating nutrient uptake to transient storage, metrics that respond to biotic characteristics like exchange coefficients and residence times may provide an advantage over those indicating relative size (A_s/A or k_1/k_2; Drummond et al. 2016). Our results suggest that the relationship between transient storage and nutrient uptake in headwaters can be temporally variable and solute-specific. Moreover, patterns can be mediated by substrate (e.g., both size and configuration; Aubeneau et al. 2014) and by biology (e.g., biofilm colonization; Aubeneau et al. 2016).

Conclusions

Billions of dollars are spent annually on efforts to restore ecological function in freshwater systems affected by agricultural and urban land use (Bernhardt et al. 2005, Mendoza-Lera and Datry 2017). We showed that substrate controlled in-stream characteristics, including biofilm development and water-residence time, which subsequently influenced removal and demand for inorganic N and P. The relationship between substrate and nutrient retention across stream sites depends largely on flow dynamics, but our results suggest that restoration efforts that enhance habitat-scale complexity or heterogeneity may provide substantial benefits between disturbance events. Changing climatic regimes are expected to increase the frequency and intensity of storms, so implementing restoration projects that maximize the capacity of streams to retain nutrients between disturbances will become increasingly important.

ACKNOWLEDGEMENTS

Author contributions: all authors contributed equally to the design and completion of this research.

This project was funded by the Notre Dame Environmental Change Initiative (ND-ECI). We thank the Notre Dame Linked Experimental Ecosystem Facility (ND-LEEF), Brett Peters, and various members of the Tank and Bolster lab groups for their assistance with establishing and completing this project. In particular, we thank Arial Shogren for her help with field work.

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