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Microbial enzyme activity, nutrient uptake and nutrient limitation in forested streams

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SUMMARY

1. We measured NH_4^+ and PO_4^{-3} uptake length (S_w), uptake velocity (V_f), uptake rate (U), biofilm respiration and enzyme activity and channel geomorphology in streams draining forested catchments in the northwestern (Northern California Coast Range and Cascade Mountains) and southeastern (Appalachian and Ouachita mountains) regions of the United States. Our goal was to use measures of biofilm enzyme activity and nutrient uptake to assess nutrient limitation in forested streams across broad regional scales.

2. Geomorphological attributes, biofilm enzyme activity and NH_4^+ uptake were significantly different among streams in the four study units. There was no study unit effect on PO_4^{-3} uptake. The proportion of the stream channel in pools, % woody debris, % canopy closure, median substrate size (d_{50}), stream width (w), stream velocity (v), discharge (Q), dispersion coefficient (D) and transient storage (A_s/A) were correlated with biofilm enzyme activity and nutrient uptake in some study units.

3. Canonical correlation analyses across study units revealed significant correlations of NH_4-V_f and PO_4-V_f with geomorphological attributes (w, d_{50}, D, % woody debris, channel slope and % pools) and biofilm phosphatase activity.

4. The results did not support our expectation that carbon processing rates by biofilm microbial assemblages would be governed by stream nutrient availability or that resulting biofilm enzyme activity would be an indicator of nutrient uptake. However, the relative abundances of peptidases, phosphatase and glycosidases did yield insight into potential N-, P- and C-limitation of stream biofilm assemblages, and our use of biofilm enzyme activity represents a novel application for understanding nutrient limitations in forested streams.

5. Regressions of V_f and U against ambient NH_4^+ and PO_4^{-3} indicated that none of our study streams was either NH_4^+ or PO_4^{-3} saturated. The Appalachian, Ouachita and Coastal streams showed evidence of NH_4^+ limitation; the Ouachita and Coastal streams were PO_4^{-3} limited. As a correlate of nutrient limitation and saturation in streams, ratios of total aminopeptidase and phosphatase activities and the ratio of NH_4-U to PO_4-U indicate these forested streams are predominantly N-limited, with only the streams draining Ouachita and Coastal catchments demonstrating appreciable levels of P-limitation.

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6. Our results comparing the stoichiometry of microbial enzyme activity with nutrient uptake ratios and with the molar ratios N and P in stream waters suggest that biological limitations are not strictly the result of stream chemistry and that the assessments of nutrient limitations in stream ecosystems should not be based on chemistry alone.
7. Our present study, along with previous work in streams, rivers and wetlands, suggests that microbial enzyme activities, especially the ratios of total peptidases to phosphatase, are useful indicators of nutrient limitations in aquatic ecosystems.

Keywords: microbial enzymes, nutrient uptake and limitation, streams

Introduction

The flow of organic matter and nutrients from catchments into the streams draining them and the biogeochemical transformations of organic matter and nutrients along flow paths are fundamental processes in streams (Hynes, 1975; Fisher, Sponseller & Heffernan, 2004). Microbial biofilms are often the primary interface for organic matter and nutrient uptake and processing in streams, and several studies have demonstrated the extent and significance of nutrient limitations on stream ecosystem functions (Davis & Minshall, 1999; Wold & Hershey, 1999; Tank & Dodds, 2003; Hoellein *et al*., 2007).

Regulation of the physical structure of streams by their catchments is often acknowledged (Frissell *et al*., 1986; Montgomery, 1999; Benda *et al*., 2004), but stream ecologists have begun only recently to link reach-scale geomorphology with the function of stream ecosystems (Fisher *et al*., 2007). The amount of catchment-derived organic matter exported to streams compared to organic matter produced by photosynthetic organisms within streams has demonstrated repeatedly the tight coupling of streams to their catchments (Vannote *et al*., 1980).

Montgomery (1999) proposed a model linking reach-scale geomorphology, governed by climate and regional geology, with ecosystem processes. His process domain concept assumes that differences in geomorphology and disturbance regimes would affect the ecological structure and function of streams. Fisher *et al*. (2007) extended this concept to include biogeochemical processes in catchment–stream ecosystems.

Respiration in aquatic systems is generally measured as O_2 consumption but also may be measured as electron transport system activity using relative levels of dehydrogenase enzymes (Broberg, 1985). Dehydrogenase activity, which can be used in both aerobic and anaerobic environments, is based on intercepting electron flow through mitochondrial and microsomal electron transport systems using a surrogate electron acceptor (Packard, 1971; Broberg, 1985). Dehydrogenase activity has been used to measure the activity of stream microbial communities and their responses to disturbances (Trevors, Mayfield & Inniss, 1982; Blenkinsopp & Lock, 1990; Hill, Herlihy & Kaufmann, 2002).

Energy flow and nutrient cycling often are linked through the microbial activity associated with biofilm on stream beds and other substrates. Microbial organisms enmeshed in this biofilm are capable of direct uptake of inorganic forms of N and P from the water column, but they also release enzymes into the biofilm for the purpose of acquiring carbon and nutrients through the degradation of organic matter (Sinsabaugh & Foreman, 2001). As a result of this linkage, nutrient uptake should be correlated with biofilm extracellular enzyme activity.

Nutrient spiralling, usually measured as uptake lengths, velocities and rates, is a frequently used approach for assessing stream ecosystem integrity. Research generally supports the view that disturbances increase uptake length and decrease uptake velocity and rate (Stream Solute Workshop, 1990; Webster *et al*., 2003; Newbold *et al*., 2006). These nutrient metrics have also been used to understand the role of catchment land use and reach-scale physicochemical factors in controlling nutrient uptake (Webster *et al*., 2003; Newbold *et al*., 2006).

Our objective was to compare biofilm enzyme activities related to N and P acquisition with N and P uptake and assess relative nutrient limitations in forested streams. We compared a suite of enzymes

produced by biofilm microbial assemblages for the acquisition of organic carbon and nutrients with ambient nutrient concentrations and nutrient uptake in those streams. Our underlying premise is that organic matter processing by biofilm microbial assemblages is so tightly governed by C : N : P ratios that carbon processing rates will be controlled directly by nutrient availability, and that nutrient uptake rates will be related to biofilm enzyme activity. We hypothesised that biofilm enzyme activity will directly reflect not only the activity of the microbial assemblage but also the nutrient status of the environment. In accordance with the ecological theory of stoichiometry, which emphasises the importance of the balance of biologically important elements for regulating an organism's response to, and regulation of, their environment (Sterner & Elser, 2002), we expected shifts in the allocation of microbial enzymes for N or P acquisition to reflect the biological demand for these nutrients, with corresponding increases or decreases in V_f, U and S_w.

Methods

Study sites

We conducted the study in 36 streams draining catchments in the northwestern and southeastern regions of the United States (Fig. 1). The catchments ranged from undisturbed, old-growth forests to those disturbed within the past 15 year by timber harvests and/or channel disturbances related to catchment slope failures (Miller, Luce & Benda, 2003).

The northwestern streams are located in the Cascade Mountains of Oregon at the H. J. Andrews Experimental Forest (six streams, 47 site-visits) and in coastal northwestern California (21 streams, 40 site-visits) (Fig. 1). The Cascades streams are underlain by volcanic bedrock and surface geologies consisting of tuff, breccia and mud and ash flows (Swanson & James, 1975). The Cascade streams drain conifer-dominated catchments and were sampled in June–August of 1999–2001. The Coastal catchments in northwestern California are underlain primarily by

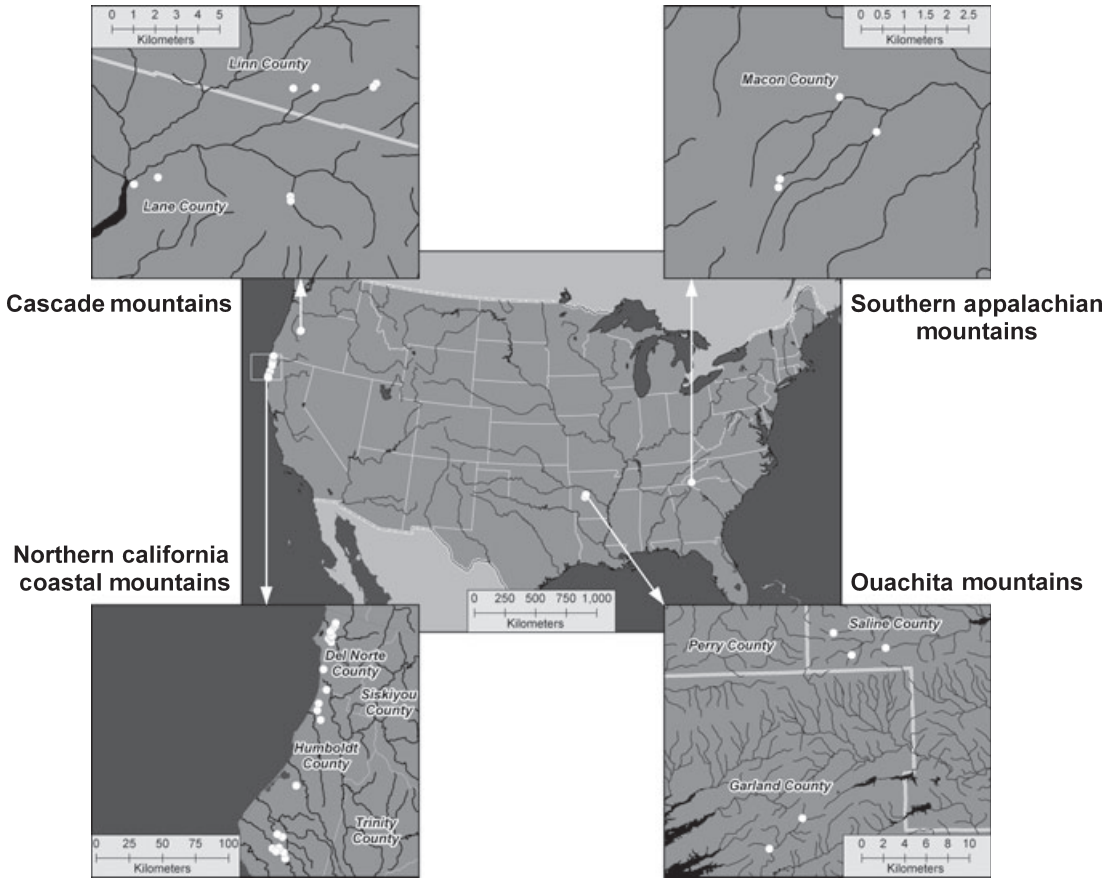

Fig. 1 Locations of the study units and streams.

uplifted Franciscan (sedimentary and volcanic) marine deposits consisting of greywacke, shale, chert and limestone (Bailey, Irwin & Jones, 1964). The Coastal streams drain conifer-dominated catchments and were sampled in July–November of 1999–2002.

The southeastern streams are located in the southern Appalachian Mountains of North Carolina at the Coweeta Hydrological Laboratory (four streams, 40 site-visits) and in the Ouachita Mountains of central Arkansas (five streams, 41 site-visits) (Fig. 1). The Appalachian and Ouachita catchments share a common pre-Cambrian bedrock geology and resulting in similar sedimentary surface geologies consisting of gneiss, chert, sandstone and dolomite (Thomas, 1985; Guccione & Zachary, 2000). The Appalachian streams drain hardwood-dominated catchments and were sampled from March–December of 1999–2001. The Ouachita streams drain mixed hardwood-conifer-dominated catchments and were sampled from February–May of 2000–03.

We estimated catchment area (A, ha) and mean annual precipitation (Ppt, cm) using the National Hydrologic Database (NHD-Plus, http://www.horizon-systems.com/nhdplus). The National Hydrologic Database depicts the presence and flow of surface waters in the United States at a nominal 1 : 100 000-scale. Elevation, land cover, air temperature and precipitation data are integrated into National Hydrologic Database.

Physicochemical measurements

We followed the US Environmental Protection Agency's Environmental Monitoring and Assessment Program (EMAP, Kaufmann *et al.*, 1999) methods for measuring riparian canopy closure (% canopy) and stream reach-scale geomorphology. These data were collected for every stream on each sampling date with the exception of the Cascade streams, in which we collected this data once each year. We collected canopy and geomorphology data from 11 evenly spaced transects along the stream reach, including channel dimensions (wetted width, w, cm; stream depth, z, cm; and stream velocity, v, cm s^{-1}), stream discharge, Q (L s^{-1}), stream slope (% slope), streambed attributes (the extent of the stream channel composed of bedrock, % bedrock; the extent of the channel covered with woody debris, % wood; the extent of the stream channel within pools, % pool) and streambed substrate size [reported as $\log_{10}$ median particle size (d_{50})].

For each sampling event, we measured the rise, plateau and decline of solute added to the stream by recording Cl^- concentration and/or specific conductivity every 30 s for the duration of the nutrient addition. Data logging continued until the specific conductivity returned to pre-release levels. We estimated dispersion coefficient (D), the rate at which a molecule of solute spreads throughout the water column, and transient storage (A_s/A), the temporary storage of solutes in water that is moving more slowly than the main stream flow, by fitting a one-dimensional solute transport model to the Cl^- tracer (measured as specific conductance) in short-term (1–4 h) solute injections of NH_4^+ and PO_4^{-3} (see *Nutrient Uptake* methods section; Stream Solute Workshop, 1990; Runkel, 1998).

Stream chemistry and biofilm collection

We collected triplicate water and biofilm (streambed surface sediments, primarily gravel) samples at each of the 11 transects prior to and immediately following the release of a nutrient and tracer solution. Water and biofilm samples were shipped on ice to the laboratory and frozen prior to processing. Frozen water samples were thawed, filtered through a 0.45 μm pore membrane and analysed immediately. All nutrient samples were analysed using a Lachat flow-injection analyser (Lachat Instruments, Milwaukee, WI, U.S.A.) with the appropriate method: NH_4^+ analysed by the phenolate method; PO_4^{-3} by the molybdate–ascorbic acid method (APHA, 1998).

Frozen biofilm samples were thawed, mixed for 1 min on a vortex mixer, and split for biofilm dehydrogenase and extracellular enzyme activities, dry weight and carbon analyses. Dry weight aliquots were oven dried (110 °C, 24 h); total C was determined by combustion using a Carla Erba (Model 1112EA) elemental analyser (Carla Erba Instrumentazione, Milan, Italy).

Biofilm respiration

We mixed duplicate aliquots for dehydrogenase activity with 2 mL of sterile H_2O and 1 mL of 0.75% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) standard, before sealing, agitating

(vortex mixer, 1 min) and incubating them (dark, 27 °C) for 3 h (Broberg, 1985; Songster-Alpin & Klotz, 1995). We terminated the incubations by adding 8 mL of methanol, then centrifuged (2000 *g*) the aliquots for 5 min, before analysing the supernatant for absorbance (428 nm) using a Perkin Elmer UV (Model Lambda 20) spectrophotometer (Perkin Elmer Life And Analytical Sciences, Inc., Wellesley, MA, U.S.A.). Aliquot absorbance was compared to a standard INT curve (prepared for each sample batch) and normalised by dry weight to calculate biofilm respiration (nmol INT g^{-1} DW h^{-1}).

Biofilm enzyme activity

We analysed the sediment samples for extracellular enzyme activity related to carbon utilisation (glycosidases), nitrogen (aminopeptidases) and phosphorus (acid phosphatase) acquisition. We measured the activity of six glycosidases (α-D-galactosidase, EC 3.2.1.22; β-D-galactosidase, EC 3.2.1.23; α-D-glucosidase, EC 3.2.1.20; β-D-glucosidase, EC 3.2.1.21; β-*N*-acetylglucosaminidase, EC 3.2.1.50; and β-D-xylosidase, EC 3.2.1.8); two aminopeptidases (L-alanine, EC 3.4.11.2; and L-leucine aminopeptidase, EC 3.4.11.1) and acid phosphatase (EC 3.1.3.2), using substrates linked to methylumbelliferyl (MUB) or coumarin (MCM) residues (Sigma–Aldrich Corporation, St. Louis, MO, U.S.A.) and the microplate protocols developed by Sinsabaugh and colleagues (Sinsabaugh *et al.*, 1997; Foreman, Franchini & Sinsabaugh, 1998; Sinsabaugh & Foreman, 2001). We prepared all substrate and reference solutions in sterile deionised water and included quadruplicate assays for each enzyme and each reference standard. Quenching, the decrease in fluorescent emissions caused by the interactions of enzyme substrates with non-reactant chemicals in the assays, was estimated by comparing the fluorescence of the supernatant of standard solutions mixed with stream sediments with that of the standard solution mixed with buffer. We incubated the microplates in the dark at 20 °C for MUB-linked substrates and for varying times at 30 °C for MCM-linked substrates. Fluorescence was measured at 60 min intervals using a BioTek (Model FLX800T) fluorometer (BioTek Instruments, Winooski, VT, U.S.A.) with an excitation wavelength of 350 nm and an emission wavelength of 450 nm. We report extracellular enzyme activity as substrate accumulated per unit sediment over time, adjusted for emission coefficients calculated from standards and corrected for quenching (nmol g^{-1} DW h^{-1}). Total enzyme activity related to C acquisition was estimated as the sum of the activities of the six glycosidases, enzyme activity related to N acquisition as the sum of the two peptidases and enzyme activity related to P acquisition as phosphatase activity.

Nutrient uptake

We measured NH_4^+ and PO_4^{-3} uptake using short-term, simultaneous additions of nutrients (K_2HPO_4, NH_4Cl) and tracer (NaCl) solutions to each 50–100- m study reach (Stream Solute Workshop, 1990). Prior to nutrient release into a stream reach, we collected water and sediment samples for ambient nutrient and biofilm respiration and enzyme activity analyses. We then added the nutrient and tracer solution at a uniform rate until a plateau in tracer concentration was detected at the most downstream station. Once plateau concentration was achieved, we collected triplicate water and sediment samples from 10 stations located every 5–10 m along the stream reach. We refrigerated the samples until they were analysed for NH_4^+ and PO_4^{-3} (APHA, 1998).

We calculated three measures of nutrient uptake: uptake length, uptake velocity and uptake rate. Nutrient uptake length (S_w, m), the average distance travelled by a nutrient molecule before being sequestered by the stream and its biota were calculated as:

$$S_w = -1/k \quad (1)$$

where k is the downstream loss rate constant (m^{-1}) calculated as the regression slope of the loss of ln-transformed nutrient concentration with stream distance (Stream Solute Workshop, 1990). Nutrient addition studies often overestimate nutrient uptake lengths, and uptake length is a linear function of nutrient additions (Mulholland *et al.*, 2002). We attempted to minimise this problem by limiting nutrient additions to 2–5 times background concentrations.

Because nutrient uptake length estimates are influenced by stream depth and velocity (Hall, Bernhardt & Likens, 2002; Hall & Tank, 2003), we calculated nutrient uptake velocity (V_f, mm min^{-1}) to allow comparisons among streams of different sizes and flows as:

$$V_f = uh/S_w \quad (2)$$

where u is the water velocity (m s^{-1}), and h is stream depth (m) (Stream Solute Workshop, 1990; Simon *et al.*, 2005). We calculated nutrient uptake rate (U, μg m^{-2} s^{-1}), the time and streambed adjusted nutrient removal rate as:

$$U = V_f C \quad (3)$$

where C is the ambient nutrient concentration (Stream Solute Workshop, 1990).

Nutrient limitation

We estimated relative NH_4^+ or PO_4^{-3} limitation based on the stoichiometry of stream chemistry, biofilm enzyme activity and nutrient U; and by the regression of nutrient V_f and U against nutrient chemistry. A comparison of the molar ratios of NH_4^+ : PO_4^{-3} for 20 streams across the United States (Webster *et al.*, 2003; Newbold *et al.*, 2006) indicated that the expected ratio is approximately 2 : 1. We classified streams as N-limited if the ratio was <1 and P-limited if the ratio was >4. Davis & Minshall (1999) demonstrated the inverse relationship of V_f and U with ambient nutrient concentrations and proposed that deviations from 16 : 1 NO_3 : PO_4 indicated relative nutrient limitation. We applied the 2 : 1 ratio and the <1 and >4 thresholds for NH_4 : PO_4 chemistry to their approach. We estimated nutrient limitations based on biofilm enzyme activity as deviations from expected 7 : 1 (<4 or >10 thresholds) ratio of peptidase to phosphatase (Chrzanowski & Kyle, 1996; Hill *et al.*, 2006; Cleveland & Liptzin, 2007). Lastly, we regressed nutrient V_f and U against nutrient concentrations to estimate relative nutrient limitations (significant negative slope of U) and saturations (significant positive slope of V_f, Hoellein *et al.*, 2007).

Statistical analyses

We compiled descriptive statistics (median and interquartile ranges) for stream chemistry, riparian canopy, stream geomorphology, biofilm enzyme activity and nutrient uptake. We tested the differences between study units using a nonparametric, one-way analysis of variance on ranked median values and the χ^2 comparison of median scores. The significance level for all analyses was $P < 0.05$. We evaluated the relationships among enzyme activity, nutrient uptake and environmental variables using Spearman rank correlation (r) to avoid problems associated with non-normal data distribution. We selected those environmental and enzyme activity variables significantly correlated with nutrient uptake for subsequent inclusion in canonical correlation analyses (CCA). The significance of the canonical correlations ($P < 0.05$) was tested using a t-test of the null hypothesis that $r_k = 0$, $t = r_k\sqrt{(1 - r_k^2)/(n - m)}$, where r_k is the canonical correlation coefficient, n the sample size and m the number of variables (Rohlf & Sokal, 1969). We used least-squares linear regression of V_f and U against ambient NH_4^+ and PO_4^{-3} to assess relative nutrient saturation and limitation. All analyses were carried out using SAS for Windows, release 9.1 (SAS Institute, Inc., Cary, CA, U.S.A.).

Results

Physicochemical characteristics of the streams

Ambient NH_4^+ and PO_4^{-3} concentrations were low in all study streams (Table 1). Median NH_4^+ and PO_4^{-3} concentrations were the lowest in the Appalachian streams (1.19 and 0.77 μg L^{-1}, respectively), and overall, there was a significant study unit effect ($P < \chi^2 <$ 0.0001 and 0.0024, Table 1). Median NH_4^+ : PO_4^{-3} ranged from 2.0 in the Appalachian streams to 5.0 in the Cascade streams and was significantly different among the study units ($P < \chi^2 < 0.0001$, Table 1). Biofilm C content ranged from 0.3% (Appalachians) to 1.2% (Cascades) and exhibited a significant study unit effect ($P < \chi^2 < 0.0001$, Table 1). Reach-scale geomorphology also varied considerably among study units (Table 1). Streams draining the California coastal catchments were wider (308 cm); those draining Cascade catchments were steeper (8.86% slope) and had more of their stream length in pools (37%); the Appalachian streams had the smallest d_{50} (1.05 $\log_{10}$ mm) and the coolest temperatures; and Ouachita streams had the largest D (0.65 m^2 s^{-1}) and A_s/A (1.33, Table 1). Only % bedrock and % canopy closure failed to exhibit significant differences among the study units (Table 1).

Canonical environmental gradients

Canonical correlation analyses revealed two significant environmental gradients related to nutrient

Table 1 Comparisons of median (interquartile range) for physicochemical attributes among streams draining catchments in the Appalachian, Ouachita, Cascade and California Coastal Mountains. The results of the χ^2 tests ($P < \chi^2$) of the nonparametric analysis of variance on median scores are presented

Variable (units)	Appalachian	Quachita	Cascade	CA Coastal	$P < \chi^2$
Catchment area (ha)	75 (18–85)	36 (18–47)	64 (64–83)	39 (10–60)	0.0006
Annual precipitation, (cm y^{-1})	200 (200–202)	145 (144–149)	218 (218–218)	195 (175–216)	<0.0001
NH_4^+ (μg L^{-1})	1.2 (0.6–2.0)	2.0 (1.4–3.4)	3.3 (2.0–4.9)	2.7 (2.2–3.9)	<0.0001
PO_4^{-3} (μg L^{-1})	0.8 (0.6–1.1)	1.5 (0.8–2.4)	1.4 (0.5–2.2)	1.4 (0.9–2.2)	0.0024
$NH_4^+ : PO_4^{-3}$	2.0 (1.7–2.4)	3.4 (2.2–5.2)	5.0 (2.2–11)	2.5 (1.7–3.8)	<0.0001
Biofilm C content (% of DW)	0.3 (0.2–0.4)	0.7 (0.5–0.9)	1.2 (0.7–1.9)	0.5 (0.4–0.6)	<0.0001
Channel as bedrock (%)	0 (0–2)	0 (0–5)	0 (0–3)	0 (0–2)	0.8546
Channel covered by wood (%)	2 (0–2)	0 (0)	0 (0–4)	0 (0)	<0.0001
Channel in pools (%)	8 (6–10)	38 (32–46)	37 (27–56)	29 (16–36)	<0.0001
Channel slope (%)	3.8 (2. 4–7.6)	0.9 (0.7–1.8)	8.9 (5.6–11)	1.9 (1.2–2.6)	<0.0001
Canopy closure (%)	94 (88–99)	94 (88–98)	87 (75–94)	94 (81–97)	0.1949
Substrate size, d_{50} ($\log_{10}$ mm)	1.0 (0.2–2.9)	3.7 (3.4–4.6)	4.0 (3.4–4.9)	4.5 (3.8–5.0)	<0.0001
Stream width, w (cm)	267 (205–287)	267 (237–291)	121 (87–253)	308 (288–373)	<0.0001
Stream depth, z (cm)	4 (4–7)	11 (8–13)	7 (5–12)	11 (8–13)	<0.0001
Stream velocity, v (cm s^{-1})	9 (6–12)	11 (6–17)	5 (3–12)	11 (8–15)	0.0419
Stream discharge, Q (L s^{-1})	10 (10–16)	30 (14–56)	6 (1–41)	43 (17–54)	<0.0001
Stream temperature, T (C)	9 (7–12)	11 (9–14)	11 (10–12)	11 (10–13)	0.0464
Dispersion coefficient, D	0.4 (0.4–0.6)	0.6 (0.6)	0.5 (0.4–0.6)	0.6 (0.5–0.6)	<0.0001
Transient storage, A_s/A	1.0 (0.8–4.0)	1.3 (1.0–2.0)	0.4 (0.3–0.8)	0.8 (0.6–1.0)	<0.0001

uptake. The first gradient (W_1) was positively correlated with w, d_{50}, D, per cent of the channel in pools and phosphatase activity and negatively correlated with channel slope and the per cent of the channel covered by wood. The second canonical gradient (W_2) was positively correlated with channel slope and phosphatase activity and negatively correlated per cent wood in the channel (Table 6). Overall, these two gradients extracted 86 and 14% of the variance in the correlations with nutrient uptake (Table 6). Several environmental variables, including stream O_2 concentrations and temperature were rejected for inclusion in the CCA because of insignificant correlations with the nutrient uptake variables.

Biofilm respiration and enzyme activity

Median biofilm respiration ranged from 241 to 384 nmol INT g^{-1} DW h^{-1} and was the lowest in the Appalachian streams. Overall, there were significant differences in biofilm respiration among the study units (Table 2). Biofilm respiration was correlated with % pool and d_{50} (Table 3). The individual median glycosidase values are presented

Table 2 Comparisons of median (interquartile range) biofilm respiration (dehydrogenase) and enzyme activity (nmol g^{-1} DW h^{-1}) among streams draining Appalachian, Ouachita, Cascade and California Coastal Mountains catchments. *β-N*-acetylglucosaminidase acts as both a glycosidase and a peptidase, and its activity was halved before inclusion in the sums of total glycosidases and total peptidases. The results of the χ^2 tests ($P < \chi^2$) of the nonparametric analysis of variance on median scores are presented

Variable	Appalachian	Ouachita	Cascade	CA Coastal	$P < \chi^2$
Dehydrogenase	241 (186–291)	367 (273–575)	384 (307–449)	315 (213–485)	<0.0001
α-D-Galactosidase	2.5 (1.7–3.9)	6.6 (2.8–15)	11 (6.7–24)	1.9 (1.1–3.2)	<0.0001
β-D-Galactosidase	3.7 (2.3–5.0)	5.8 (2.4–7.3)	6.0 (3.2–12)	1.8 (1.0–3.4)	<0.0001
α-D-Glucosidase	1.4 (0.6–2.6)	4.2 (1.6–10)	5.5 (3.6–7.6)	0.8 (0.4–1.3)	<0.0001
β-D-Glucosidase	62 (45–101)	92 (47–180)	170 (101–238)	15 (7.8–27)	<0.0001
β-D-Xylosidase	13 (7.1–18)	10 (4.6–18)	16 (9.1–22)	2.1 (1.2–3.6)	<0.0001
β-*N*-acetylglucosaminidase	15 (10–25)	32 (15–52)	64 (40–100)	11 (3.5–23)	<0.0001
Total glycosidases	99 (68–146)	147 (77–291)	261 (153–328)	31 (21–59)	<0.0001
L-Alanine aminopeptidase	9.7 (5.4–12)	12 (7.0–16)	11 (4.3–19)	3.9 (0.6–14)	0.0024
L-Leucine aminopeptidase	6.9 (4.2–11)	9.2 (6.2–15)	8.5 (2.1–13)	3.5 (1.2–8.5)	0.0166
Total aminopeptidases	96 (74–159)	200 (95–307)	390 (259–543)	69 (37–134)	<0.0001
Acid phosphatase	30 (20–44)	116 (50–242)	55 (29–108)	17 (9.0–48)	<0.0001

Table 3 Spearman correlation coefficients (r) for the comparisons of dehydrogenase, total glycosidase, total peptidase and acid phosphatase activity in forested streams with selected physicochemical variables. (—) Indicates correlations with $P > 0.05$

Variable	Total dehydrogenase	Total glycosidases	Total peptidases	Acid phosphatase
NH_4	—	—	—	—
PO_4	—	—	—	—
Channel as bedrock	—	—	—	—
Channel covered by wood	—	0.22	0.17	—
Channel in pools	0.32	—	0.27	0.27
Channel slope	—	0.31	0.23	—
Canopy closure	—	—	—	—
Substrate size	0.31	—	—	—
Stream width	—	−0.39	−0.27	−0.26
Stream depth	—	—	—	—
Stream velocity	—	−0.20	−0.23	—
Stream discharge	—	−0.32	−0.21	—
Dispersion coefficient	—	—	—	0.31
Transient storage	—	—	—	0.18

in Table 2. The sum of glycosidase activity was the lowest in the streams draining the California coastal catchments (31 nmol g^{-1} DW h^{-1}) and was correlated with % woody debris, channel slope, w, v and Q (Table 3). Similarly, median alanine and leucine aminopeptidase activities and total aminopeptidase activity were the lowest in the California Coastal streams (Table 2). Total peptidase activity was correlated with % woody debris, % pool, slope, w, v and Q (Table 3). Median acid phosphatase activity ranged from 69 nmol g^{-1} DW h^{-1} in the coastal streams to 390 nmol g^{-1} DW h^{-1} in the Cascade Mountain streams (Table 2) and was correlated with % pool, w, D and A_s/A (Table 3). All biofilm respiration and enzyme activities exhibited significant study unit effects ($P < \chi^2 < 0.0001$ and 0.0166, Table 2).

Nutrient uptake

Median NH_4-S_w ranged from 42 to 143 m and differed significantly among the study units ($P < \chi^2 < 0.0075$, Table 4). NH_4-S_w was correlated with % pool and phosphatase activity (Table 5). Median NH_4-V_f was the lowest in the Appalachian streams (2.12 m d^{-1}) and exhibited a significant study unit effect ($P < \chi^2 < 0.0028$, Table 4). NH_4-V_f was correlated with % woody debris, % pool, slope, d_{50}, w and D (Table 5), and with the first canonical environmental gradient (W_1, Table 6). Median NH_4-U ranged from 9.69 to 150 μg m^{-2} h^{-1} and was the lowest in the streams draining the Appalachian catchments ($P < \chi^2 < 0.0007$, Table 4). NH_4-U was correlated with % woody debris, % pool, slope, d_{50} and w (Table 5).

Median PO_4-S_w ranged from 106 to 353 m but failed to exhibit a significant unit effect ($P < \chi^2 < 0.2355$, Table 4). PO_4-S_w was correlated with % woody debris, w, z, Q, D and phosphatase activity (Table 5). Median PO_4-V_f ranged from 1.36 to 2.23 m d^{-1} but was not significantly different among the study units ($P < \chi^2 < 0.5024$, Table 4). PO_4-V_f was correlated with d_{50}, w and D (Table 5), and with the first canonical environmental gradient (W_1, Table 6). Median PO_4-U ranged from 8.89 to 27.7 μg m^{-2} h^{-1} and was not

Table 4 Comparisons of median (interquartile range) for NH_4^+ and PO_4^{-3} uptake length (S_w, m), uptake velocity (V_f, m d^{-1}) and uptake rate (U, μg m^{-2} h^{-1}) among streams draining catchments in the Appalachian, Ouachita, Cascade and California Coastal Mountains. The χ^2 tests ($P < \chi^2$) of the nonparametric analysis of variance on median scores are presented

Variable	Appalachian	Ouachita	Cascade	CA Coastal	$P < \chi^2$
NH_4^+					
-S_w	143 (33–330)	42 (26–84)	94 (44–253)	135 (47–223)	0.0075
-V_f,	2.1 (0.8–4.6)	13 (7.2–26)	7.2 (0.7–16)	3.8 (1.9–14)	0.0028
-U	9.7 (7.0–44)	225 (92–385)	113 (22–370)	150 (42–704)	0.0007
PO_4^{-3}					
-S_w	136 (76–272)	110 (73–228)	106 (66–256)	353 (98–813)	0.2355
-V_f,	1.4 (0.7–2.7)	2.2 (1.4–6.0)	1.9 (0.5–2.7)	1.8 (0.8–5.3)	0.5024
-U	8.9 (4.9–19)	21 (12–49)	21 (7.1–32)	28 (9.0–69)	0.1985

Table 5 Spearman correlation coefficients (r) for the comparisons of NH_4^+ and PO_4^{-3} uptake length (S_w), uptake velocity (V_f) and uptake rate (U) in forested streams in the four study units with median substrate size, dispersion coefficient, transient storage and biofilm enzyme activity (dehydrogenase, total glycosidase, total peptidase, acid phosphatase). (—) Indicates correlations with $P > 0.05$. Variables that are derived from other variables are indicated as not applicable (na)

	NH_4			PO_4		
	S_w	V_f	U	S_w	V_f	U
NH_4	—	—	na	—	—	0.29
PO_4	—	—	—	—	—	na
Channel as bedrock	—	—	—	—	—	—
Channel covered by wood	—	−0.23	−0.34	−0.28	—	—
Channel in pools	−0.26	0.28	−0.25	—	—	0.22
Channel slope	—	−0.22	−0.25	—	—	−0.21
Canopy closure	—	—	—	—	—	—
Substrate size	—	0.29	0.45	—	0.29	0.26
Stream width	—	0.42	0.48	0.29	0.24	—
Stream depth	—	na	na	0.25	na	na
Stream velocity	—	na	na	0.28	na	na
Stream discharge	—	na	na	0.33	na	na
Dispersion coefficient	—	0.23	—	—	0.26	0.34
Transient storage	—	—	—	—	—	—
Dehydrogenase activity	—	—	—	—	—	—
Total glycosidase activity	—	—	—	—	—	—
Total peptidase activity	—	—	—	—	—	—
Acid phosphatase activity	−0.24	—	—	−0.24	—	—

Table 6 Canonical correlations of NH_4 and PO_4 uptake velocity (V_f) in forested streams with the canonical environmental gradients (W_1, W_2). The model includes only those environmental and biofilm enzyme variables demonstrating significant correlations with NH_4 and PO_4 uptake metrics (Table 5). Significant correlations ($P < 0.05$) are in bold

Variable	W_1	W_2
Channel covered by wood	**−0.56**	**0.39**
Channel in pools	**0.31**	−0.22
Channel slope	**−0.39**	**0.45**
Substrate size	**0.60**	0.13
Stream width	**0.69**	−0.24
Dispersion coefficient	**0.55**	0.05
Transient storage	−0.24	−0.08
Acid phosphatase activity	**0.43**	**0.66**
NH_4-V_f	**0.52**	−0.19
PO_4-V_f	**0.54**	0.18
% Variance extracted	86	14

significantly different among the streams draining the four study units ($P < \chi^2 < 0.1985$, Table 4). PO_4-U was correlated with NH_4, % pool, slope, d_{50} and D (Table 5).

Nutrient limitation

We estimated nutrient limitation using three stoichiometric measures. Stream water NH_4 : PO_4 indicated that some degree of nutrient limitation was evident in all of the study units. N-limitation ranged from 5% of the sampling events in the Ouachita streams to 38% of the site-visits for the Appalachian streams; P-limitation ranged from 2% of the Appalachian stream sampling events to 49% of the site-visits in the Cascades (Table 7). Nutrient limitations estimated from the stoichiometry of NH_4^+ and PO_4^{-3} uptake rates suggested a much greater extent of nutrient limitation than did stream chemistry. Nutrient uptake rates indicated N-limitation on 69–80% of the stream sampling events, with only 5–20% of those visits indicating P-limitation (Table 7). Nutrient limitation based on the stoichiometry of biofilm enzyme activities was more similar to estimates based on nutrient uptake than to those based on stream chemistry. Biofilm enzyme activity stoichiometry indicated that N-limitation was prevalent (56–75% of the sampling events) in all of the study units; and P-limitation was a much less common occurrence (Table 7). Biofilm enzyme activity also revealed some degree of C-limitation (10–38% of the site-visits) in all of the study units, but especially in the Appalachian and Ouachita streams (Table 7).

Regression analyses of NH_4- and PO_4-V_f and U against stream nutrient availability indicated that

Table 7 Per cent of the sampling events in Appalachian, Ouachita, Cascade and Coastal Mountain streams that indicated N- or P-limitations based on biofilm enzyme activities, nutrient uptake rates and stream chemistry. Also listed is the per cent of stream sites that appear to be limited by C availability based on biofilm enzyme activity

Limitation	Appalachian	Ouachita	Cascade	Coastal
Enzyme activity				
N-limited	75	61	62	56
P-limited	3	29	0	24
C-limited	38	20	15	10
Nutrient uptake rates				
N-limited	80	78	83	69
P-limited	5	20	13	20
Stream chemistry				
N-limited	38	5	15	27
P-limited	2	44	49	22

none of the streams in any of the study units was nutrient saturated, but several streams exhibited either N- or P-limitation. Appalachian, Ouachita and Coastal streams all exhibited N-limitation; Ouachita and Coastal streams appeared to also be P-limited (Table 8).

Table 8 Log–log regression models for NH_4^+ and PO_4^{-3} uptake velocity (V_f) and uptake rate (U) against ambient NH_4^+ and PO_4^{-3} concentrations in the study streams. Significant negative slopes for the $V_f = NH_4^+$ or PO_4^{-3} models indicate NH_4^+ or PO_4^{-3} saturation. Significant positive slopes for the $U = NH_4^+$ or PO_4^{-3} models indicate NH_4^+ or PO_4^{-3} limitation. See Discussion for details

Unit	Model	r^2	$P < F$
Nutrient saturation models			
Appalachian	NH_4^+-$V_f = -0.008 \cdot NH_4^+ + 0.294$	0.0011	0.919
Ouachita	NH_4^+-$V_f = 0.039 \cdot NH_4^+ + 0.722$	0.0613	0.438
Cascade	NH_4^+-$V_f = 0.027 \cdot NH_4^+ + 0.424$	0.0344	0.585
Coastal	NH_4^+-$V_f = 0.008 \cdot NH_4^+ + 0.528$	0.0765	0.213
Appalachian	PO_4^+-$V_f = -0.187 \cdot PO_4^+ - 1.493$	0.0491	0.489
Ouachita	PO_4^+-$V_f = 0.048 \cdot PO_4^+ - 1.674$	0.0900	0.343
Cascade	PO_4^+-$V_f = -0.768 \cdot PO_4^+ - 0.373$	0.2830	0.092
Coastal	PO_4^+-$V_f = 0.027 \cdot PO_4^+ - 1.700$	0.0576	0.282
Nutrient limitation models			
Appalachian	NH_4^+-$U = 0.980 \cdot NH_4^+ + 1.222$	0.6000	0.003
Ouachita	NH_4^+-$U = 0.410 \cdot NH_4^+ + 3.731$	0.5335	0.007
Cascade	NH_4^+-$U = 0.284 \cdot NH_4^+ + 3.085$	0.2336	0.132
Coastal	NH_4^+-$U = 0.071 \cdot NH_4^+ + 4.405$	0.3527	0.004
Appalachian	PO_4^+-$U = 0.419 \cdot PO_4^+ + 1.978$	0.1912	0.155
Ouachita	PO_4^+-$U = 0.144 \cdot PO_4^+ + 2.617$	0.6071	0.003
Cascade	PO_4^+-$U = 0.120 \cdot PO_4^+ + 2.670$	0.0112	0.757
Coastal	PO_4^+-$U = 0.094 \cdot PO_4^+ + 3.012$	0.4202	0.001

Discussion

Our measures of NH_4-S_w are within reported ranges for small streams. Several recent studies reported substantially shorter minimum NH_4-S_w (5–50 m), but they also reported maximum NH_4-S_w exceeding our estimates (Hall *et al.*, 2002; Mulholland *et al.*, 2002; Webster *et al.*, 2003; Simon *et al.*, 2005; Hoellein *et al.*, 2007). Similarly, our estimates of NH_4-V_f and NH_4-U are on par with estimates from other small, forested streams (Hall *et al.*, 2002; Mulholland *et al.*, 2002; Webster *et al.*, 2003; Simon *et al.*, 2005; Newbold *et al.*, 2006; Bukaveckas, 2007; Hoellein *et al.*, 2007). Our estimates of biofilm enzyme activities are within the ranges reported from small streams in North America and Europe (Sinsabaugh & Linkins, 1988; Blenkinsopp & Lock, 1990; Sinsabaugh *et al.*, 1991; Songster-Alpin & Klotz, 1995; Hill *et al.*, 2002; Rulik & Spacil, 2004). Low biofilm respiration and enzyme activities may reflect the low nutrient concentrations and dense canopy closure of our study streams.

With few exceptions, our estimates of PO_4-S_w are longer than most reported in recent literature. Estimated PO_4-S_w in forested streams ranges from 2 to 156 m (Hall *et al.*, 2002; Mulholland *et al.*, 2002; Valett, Crenshaw & Wagner, 2002; Webster *et al.*, 2003; Bukaveckas, 2007; Hoellein *et al.*, 2007). The few exceptions that were longer than our estimates of PO_4-S_w come from wilderness, headwater streams in the western United States (Davis & Minshall, 1999) and from nutrient-enriched agricultural streams in the Midwestern United States (Bernot *et al.*, 2006). Despite these differences in PO_4-S_w, our estimates of PO_4-V_f and PO_4-U are within the range of values reported by these authors. Some of the differences in NH_4-S_w and PO_4-S_w among the studies cited may be attributable to the overestimation of uptake length differences inherent in nutrient addition studies (Mulholland *et al.*, 2002; Payn *et al.*, 2005; Earl, Valett & Webster, 2007; Hanafi *et al.*, 2007).

Studies examining factors affecting N and P uptake in streams have provided mixed results. Uptake in 11 streams spanning a range of climatic and hydrological conditions showed no significant correlations between NH_4^+ uptake and stream temperature, stream width, depth or discharge, transient storage or stream metabolism, but good correspondence between estimated inorganic N demands and estimates of assimilative N uptake (Webster *et al.*, 2003). In Michigan

headwater streams, positive correlations occurred between NH_4^+-V_f and PO_4^{-3}-V_f, and the proportion of the streambed composed of large inorganic substrates (Hoellein *et al.*, 2007). This finding, although supporting the results of greater nutrient uptake on coarser grained sediments (Aumen, Hawkins & Gregory (1990), runs counter to a laboratory finding (Munn & Meyer, 1990) of the greatest PO_4^{-3} sorption by fine-grained sediments from two streams included in our study (Hugh White Creek, North Carolina and the stream draining Catchment 2, Oregon). Greater P uptake rates were associated with fine-grained streambed sediments in an urban stream (Ryan, Packman & Kilham, 2007), but Marti & Sabater (1996) reported inconclusive results relating NH_4^+ and PO_4^{-3} uptake lengths and rates with sediment size classes in two Mediterranean streams. The correlation of our uptake data with d_{50} and with the first canonical environmental gradient suggests a positive relationship between them. Several studies also demonstrate positive correlations between nutrient uptake and coarse woody debris in the stream channel (e.g., Aumen *et al.*, 1990; Munn & Meyer, 1990; Hoellein *et al.*, 2007). The correlations of our NH_4^+ and PO_4^{-3} uptake inversely correlated with woody debris in half the cases. We found little correlation of uptake metrics with stream chemistry, A_s/A or biofilm enzyme activities (except for a weak correlation of phosphatase activity with S_w) (Table 5).

While much has been written about NH_4^+ and PO_4^{-3} uptake, very few studies discuss the stoichiometry of these nutrients. Webster *et al.* (2003) reported NH_4 : PO_4 ranging from 0.1 to 10, with an average of 1.7 for 10 North American streams. Similarly, Newbold *et al.* (2006) reported NH_4 : PO_4 ranging from 0.1 to 12, with an average of 0.8 for 10 streams in the Hudson River valley of New York. Neither of these studies discusses the significance of NH_4 : PO_4, nor do they attempt to classify nutrient limitations in these streams according this ratio. Our study directly compares these biologically active nutrients and assesses the potential for nutrient limitation of stream ecosystem function. The significance of NH_4 : PO_4 may be as it relates to biological measures of nutrient uptake and biological activity (Simon *et al.*, 2005). Our two measures of biological N- and P-limitation based on the stoichiometry of nutrient uptake and biofilm enzyme activity revealed significantly more nutrient limitation than was expected based on stream chemistry.

Microbial nutrient demand is determined by the elemental stoichiometry of microbial biomass in relation to environmental nutrient availability. Microbial respiration and nutrient immobilisation increase the N and P content of organic matter as it decomposes, eventually creating aquatic sediment with mean N : P of 7 : 1 (Hill *et al.*, 2006, 2010). The streams in our study are divided into three classes based on their relative biofilm enzyme activities: (i) reaches in which peptidase activity greater than phosphatase or glycosidase activities; (ii) reaches in which phosphatase activity greater than peptidase or glycosidase activities and (iii) reaches in which glycosidase activity greater than either peptidase or phosphatase activities. Stream reaches exhibiting peptidase dominance are presumed to be relatively N-limited. Leucine aminopeptidase is one of the most frequently measured extracellular enzymes (along with phosphatase and glucosidases, Sinsabaugh & Foreman, 2001). Several studies demonstrate increasing peptidase activity with declining N availability (Boschker & Cappenberg, 1998; Montuelle & Volat, 1998; Ainsworth & Goulder, 2000). The second class is those stream reaches dominated by phosphatase activity. Increase in phosphatase activity in response to declining or limiting P has been demonstrated in numerous aquatic environments (Cotner & Wetzel, 1991; Kang & Freeman, 1999; Shackle, Freeman & Reynolds, 2000; Wright & Reddy, 2001). The third class of streams is those dominated by glycosidase activity and presumed to be relatively C-limited. Carbon supply is a well-investigated constraint on microbial productivity and biofilm enzyme activity (Sinsabaugh *et al.*, 1997; Shackle *et al.*, 2000; Burns & Ryder, 2001; Sinsabaugh & Foreman, 2001; Harbott & Grace, 2005; Kang *et al.*, 2005). The streams in this study were predominantly N- and C-limited, with only those streams draining Ouachita and Coastal catchments exhibiting P-limitation (Table 7), a finding similar to that reported by Tank & Dodds (2003) from their survey of North American streams.

Differential N- and P-limitation may account for some of the differences in nutrient uptake reported in the literature. The three biological approaches we used for elucidating nutrient limitation suggest similar but not identical results. Davis & Minshall (1999) demonstrated the inverse relationship of V_f and U with ambient nutrient concentrations and suggested that these measures are indicators of relative nutrient

limitation in streams. They argued that the ratio of reach-scale N to P uptake rates provides an estimate of the relative nutrient requirements of the stream reach, and that ratios deviating from an assumed Redfield ratio (16N : 1P) indicate either P-limitation (NH_4-U: PO_4-U < 16 : 1) or N-limitation (NH_4-U: PO_4-U > 16 : 1). NH_4-U: PO_4-U in our study streams suggests that most streams were N-limited (Table 7). Hoellein *et al.* (2007) expanded on the nutrient limitation aspect of nutrient uptake metrics. They proposed that the regression of V_f and U against ambient nutrient concentrations yields information on nutrient limitations. In these analyses, a negative slope for V_f indicates that nutrient uptake is approaching saturation; a positive slope for U indicates nutrient uptake is nutrient limited. Regression analyses of our streams indicated that none of our study streams was either NH_4^+ or PO_4^{-3} saturated; the Appalachian, Ouachita and Coastal Mountain streams showed evidence of NH_4^+ limitation; Ouachita and Coastal Mountain streams were PO_4^{-3} limited (Table 8, Fig. 2).

Our estimates of overall biofilm enzyme activity, and its relative activities among the various enzymes, are similar to values reported for streams and rivers (Sinsabaugh & Findlay, 1995; Fischer *et al.*, 2005) and from other aquatic ecosystems (Boschker & Cappenberg, 1998; Ainsworth & Goulder, 2000; Burns & Ryder, 2001; Sinsabaugh & Foreman, 2001; Harbott & Grace, 2005). Most authors report enzyme activities dominated by β-glucosidase, leucine aminopeptidase and phosphatase, as was generally the case for the streams we studied.

We set out to compare biofilm enzyme activity with nutrient uptake in the forested streams under the premise that organic matter processing by biofilm microbial assemblages is controlled by nutrient availability. Our expectation that nutrient uptake in these streams is tightly coupled to biofilm enzyme activity is not supported by our data. We further hypothesised that the relative abundances of peptidases, phosphatase and glycosidases would yield insight into potential N-, P- and C-limitation, and our results generally agree with the estimates of nutrient limitation based on V_f and U. Our findings are supportive of ecological stoichiometric theory, which emphasises the importance of the balance of biologically important elements for regulating an organism's response to, and regulation of, their environment (Sterner & Elser, 2002).

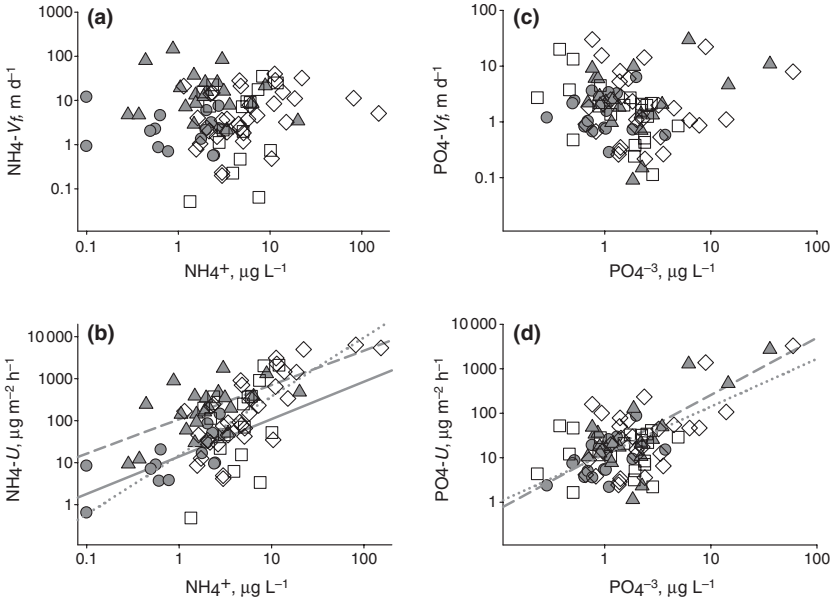

Fig. 2 Regression plots of (a) NH_4-V_f, (b) NH_4-U, (c) PO_4-V_f and (d) PO_4-U against ambient NH_4^+ and PO_4^{-3} concentrations for Appalachian (solid circles, solid line), Ouachita (solid triangles, dashed-dotted line), Cascade (open squares, dashed line) and Coastal (open diamonds, dotted line) Mountain streams. Regression lines are plotted only for significant regression. The lack of significant inverse relationships in panels 'a' and 'c' suggest than none of the study streams was NH_4^+ or PO_4^{-3} saturated. The positive regression slopes in panel 'b' indicates that Appalachian, Ouachita and Coastal Mountain streams were NH_4^+ limited; the regression slopes in panel 'd' indicate PO_4^{-3} limitation in Ouachita and Coastal Mountain streams. See regression results in Table 7.

Biofilm enzyme activity in streams and its correlation with physicochemical attributes and nutrient uptake were previously unexamined, especially spanning broad, regional scales (Blenkinsopp & Lock, 1990; Sinsabaugh *et al.*, 1991; Rulik & Spacil, 2004; Harbott & Grace, 2005). Hill *et al.* (2006, 2010) provided the only other examples of using enzyme activities in a large regional study. They examined extracellular enzyme activity on sediments and its relationship to water and sediment chemistry, and to catchment-scale stressors, to assess nutrient limitations in Laurentian Great Lakes coastal wetlands and in large, flood plain rivers of the Mississippi River basin. Our results comparing the stoichiometry of microbial enzyme activity with nutrient uptake ratios, and with the molar ratios N to P in stream waters, indicate that biological limitations are not strictly the result of stream chemistry and that assessments of nutrient limitations in stream ecosystems should not be based on chemistry alone. Our present study, along with previous work in streams, rivers and wetlands, suggests that microbial enzyme activities related to nutrient acquisition may be useful for assessing the nutrient status of aquatic ecosystems.

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