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Chronic catchment nitrogen enrichment and stoichiometric constraints on the bioavailability of dissolved organic matter from leaf leachate

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SUMMARY

1. Chronic nitrogen (N) deposition may alter the bioavailability of dissolved organic matter (DOM) in streams by multiple pathways. Elevated N deposition may alter the nutrient stoichiometry of DOM as well as nutrient availability in stream water.

2. We evaluated the influence of a decadal-scale experimental N enrichment on the relative importance of DOM nutrient content and inorganic nutrient availability on the bioavailability of DOM. We measured the consumption of dissolved organic carbon (DOC) and changes in nutrient concentration, DOM components and enzyme activity in a bottle incubation assay with different DOM and nutrient treatments. To evaluate the effect of DOM stoichiometry, we used leaf leachates of different carbon/N/phosphorus (C : N :P) ratio, made from leaf litter sourced in the reference and N-enriched catchments at the Bear Brook Watershed in Maine (BBWM). We also manipulated the concentration of inorganic N and P to compare the effect of nutrient enrichment with DOM stoichiometry.

3. DOC from the N-enriched catchment was consumed 14% faster than that from the reference catchment. However, mean DOC consumption for both leachates was more than doubled by the simultaneous addition of N and P, compared to controls, while the addition of N or P alone increased consumption by 42 and 23%, respectively. The effect of N and/or P enrichment consistently had a greater effect than DOM source for all response variables considered.

4. We subsequently conducted DOC uptake measurements using leaf leachate addition under ambient and elevated N and P in the streams draining the reference and N-enriched catchments at BBWM. In both streams, DOC uptake lengths were shorter when N and P were elevated.

5. Although both DOM stoichiometry and inorganic nutrient availability affect DOM bioavailability, N and P co-limitation appears to be the dominant driver of reach-scale processing of DOM.

Keywords: bioavailability, dissolved organic matter, nitrogen deposition, nutrient limitation, streams

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Introduction

Freshwater ecosystems process considerable amounts of organic carbon (C) exported from terrestrial ecosystems (Cole *et al*., 2007), and aquatic microbes are responsible for much of this processing (Cotner & Biddanda, 2002). Most terrigenous C enters aquatic ecosystems as dissolved organic matter (DOM) which can play a critical role in freshwater ecosystems by fuelling microbial production in estuaries (Raymond & Bauer, 2000), lakes (Cole *et al*., 2006) and streams (Bott, Kaplan & Kuserk, 1984; Hall & Meyer, 1998) and subsequently feed higher trophic levels in these ecosystems (e.g. Hall & Meyer, 1998). DOM is a complex pool of compounds with varying lability (Kaplan *et al*., 2008). Consequently, the chemical composition of DOM influences its degradation and associated microbial productivity (Meyer, 1994). A key component of organic matter quality is the relative balance between C and nutrients (i.e. nitrogen N and phosphorus P) within organic compounds; lower ratios are typically associated with higher quality and greater organic matter consumption (Enriquez & Duarte, 1993; Kroer, 1993).

The nutrient content of DOM as well as inorganic nutrient availability of the environment may affect microbial use and degradation of DOM (Marschner & Kalbitz, 2003). Nutrient limitation of heterotrophic microbial biomass and activity is supported by many studies that have found that experimental addition of inorganic nutrients resulted in increased microbial biomass (e.g. Pace & Cole, 1996) and respiration, as well as accelerated organic matter decomposition rates (Howarth & Fisher, 1976; Gulis & Suberkropp, 2003; Stelzer, Heffernan & Likens, 2003). Stimulation of DOM processing by the environmental supply of inorganic nutrients has also been demonstrated by models (Sinsabaugh *et al*., 1997; Thingstad *et al*., 1999) and in laboratory bioassays (Zweifel *et al*., 1995; Wikner, Cuadros & Jansson, 1999). A need to address the interaction between organic and inorganic nutrient availability in influencing organic matter processing has been emphasised in terrestrial systems (e.g. Melillo, Aber & Muratore, 1982); understanding DOM processing in aquatic systems requires a similar approach (Donahue *et al*., 1998; McKnight *et al*., 2001).

Microbes can acquire nutrients directly from inorganic pools or they can deploy a suite of extracellular enzymes that liberate N and P embedded within organic molecules for subsequent microbial uptake (Sinsabaugh *et al*., 2010); such microbial processing represents a key linkage point for C, N and P cycling (McGill & Cole, 1981; Falkowski, Fenchel & Delong, 2008). For example, when inorganic P is scarce, microbes commonly increase phosphatase production to acquire P from organic pools (Scott *et al*., 2009). Production of enzymes for N and P acquisition incurs a cost of reduced allocation towards C acquisition, setting up a dynamic system in which microbes continually shift resource allocation towards acquisition of C, N and P from multiple sources (Sinsabaugh *et al*., 1997; Sinsabaugh & Follstad Shah, 2010). As such, patterns of enzymatic allocation can explain the shifts in biogeochemical cycling and provide insights into constraints on the microbial response to shifting availability of multiple elements.

Anthropogenic activity often shifts the elemental balance of nutrient availability in inorganic and organic pools with consequences for decomposition and biogeochemical cycling. A widespread example is atmospheric N deposition, which can lower DOM C : N (McDowell *et al*., 1998) and increase N concentration in terrestrial vegetation (Aber *et al*., 1998) and inorganic N availability in aquatic ecosystems (Williams *et al*., 1996; Navrátil *et al*., 2010). Increased organic and inorganic N availability may result in enhanced degradability of organic matter; however, such a shift in nutrient stoichiometry may also induce microbial P limitation that constrains DOM degradation. Increased P limitation of primary producers with increasing chronic N deposition has been observed in lakes (Elser *et al*., 2009), but it is largely unexplored in regard to DOM utilisation.

In this context, we examined the effects of chronic catchment N enrichment on DOM bioavailability through the alteration in DOM nutrient content and inorganic nutrient availability at a whole-catchment experimental site, the Bear Brook Watershed in Maine (BBWM). The BBWM is a paired catchment experiment comprised of a reference catchment and a contiguous treated catchment subjected to 22 years of elevated N deposition and acidification at the time of this study. The long-term treatment has led to elevated N in terrestrial vegetation (Elvir *et al*., 2005; Hunt, Ohno & Fernandez, 2008) and in stream water (Navrátil *et al*., 2010). We conducted laboratory bioassays in which we manipulated DOM derived from leaves collected in the reference and treatment catchments, as well as water chemistry (i.e. inorganic N and P) and measured DOM (as DOC) consumption and changes in DOM components, nutrient concentrations and microbial enzyme activity. We followed this experiment with whole-stream additions of DOM in the reference and N-enriched catchments under ambient and elevated stream water N and P. We expected that prolonged N addition would alleviate N limitation of microbial DOM consumption by enhancing N content in DOM and in stream water, but that the stimulatory effect of increasing N would become limited by P availability.

Methods

Study site description

The BBWM is located in eastern Maine, U.S.A., in Township 28 (44°52'N, 68°06'W), and is described in detail by Norton *et al.* (2010). The BBWM site was chosen based on the similar hydrology and catchment characteristics of the two catchments prior to catchment-scale manipulation of N and sulphur (S) inputs. West Bear Brook and East Bear Brook, form a paired catchment complex with first-order streams draining 11.0 and 10.3 ha, respectively. West Bear Brook is the treatment (TRT) catchment that has received bimonthly applications of $(NH_4)_2SO_4$ by helicopter since November 1989 at a dose of 25.2 kg N ha^{-1} $year^{-1}$, while East Bear Brook has remained untreated, as a reference (REF) catchment. In both streams, NO_3-N concentrations are low (*c.* <30 μg L^{-1}) during the growing season (June–September), but during the remainder of the year, NO_3-N in the TRT stream is about 50 times higher than in the REF stream (Navrátil *et al.*, 2010). DOC concentration is typically slightly higher in the reference stream than in the treatment stream (mean concentration of 1.9 and 1.5 mg L^{-1}, respectively), and there are no long-term trends in either stream (Navrátil *et al.*, 2010). Since initiation of the treatment, mean annual pH has been 5.48 in the REF stream and 5.25 in the TRT stream (Laudon & Norton, 2010). The N enrichment and acidification in the TRT catchment have resulted in elevated N and P foliar concentrations compared to REF foliage (Elvir *et al.*, 2005). Vegetation at BBWM is northern hardwoods at lower elevations (mostly American beech, *Fagus grandifolia*, and sugar maple, *Acer saccharum*) and softwood stands at higher elevation (dominated by red spruce, *Picea rubens*, and balsam fir, *Abies balsamea*). Annual precipitation ranges from 90–140 cm and temperature from −30 °C in the winter to 35 °C in the summer.

Incubation bottle preparation

We used a full-factorial design to evaluate the effects of DOM source (i.e. catchment vegetation), water/microbe origin (stream) and inorganic nutrient (N and P) amendment on microbial degradation of DOM (measured as DOC). To do this, a water and microbe mixture from each stream (REF and TRT) was treated with DOM leached from leaves from either catchment (DOM_{REF} and DOM_{TRT}) and incubated under ambient or elevated levels of inorganic N and/or P (2 streams × 2 DOM sources × 4 nutrient treatments = 16 treatments; Table 1). The water/microbe mixes from each stream were prepared by filtering (0.7 μm, Whatman GF/F) 20 L of stream water into a carboy and dosing it with 10 mL of a microbial slurry collected from the same stream. Microbial slurries were made by scrubbing three rocks from the stream bed in 100 mL of stream water using a sterile toothbrush. Water in each carboy was then divided into two large plastic jugs, and each jug was amended with one of the prepared leachates (described below) to a target of 10 mg C L^{-1}. Each of the four solutions was then distributed into four separate 2-L flasks which received either a nutrient amendment including N (as KNO_3), P (as KH_2PO_4), combined N and P or deionised water (ambient). Each of the 2-L flasks was then subdivided among 4 replicate 500-mL glass bottles which were loosely capped and incubated in the dark at 14 °C for 30 days (n = 64 total bottles). The experiment was conducted during the summer when NO_3-N concentration was at a seasonal low level in both streams (TRT = 17 μg NO_3-N L^{-1} and REF = 4 μg NO_3-N L^{-1}). Target concentration for N and P (as soluble reactive P, SRP) enrichments were 350 and 100 μg L^{-1}, respectively. Each bottle was inverted several times every 2 days to homogenise the samples and maintain aerobic conditions in the bottles (confirmed by periodic measurements using a Hach HQ40d multi-parameter meter with a luminescent dissolved oxygen probe).

Table 1 Treatment design for the laboratory experiment. REF and TRT are mixtures of stream water and microbes from the reference and treatment streams, respectively. DOM_{REF} and DOM_{TRT} are DOM derived from leaves sourced from the reference and treatment catchments, respectively (n = 4/cell). +Nitrogen and + Phosphorus are inorganic nutrient amendments

	Stream source (water and microbe)			
DOM source	REF		TRT	
DOM_{TRT}	Control	+ Nitrogen	Control	+ Nitrogen
	+ Phosphorus	+ Nitrogen + Phosphorus	+ Phosphorus	+ Nitrogen + Phosphorus
DOM_{REF}	Control	+ Nitrogen	Control	+ Nitrogen
	+ Phosphorus	+ Nitrogen + Phosphorus	+ Phosphorus	+ Nitrogen + Phosphorus

DOM leachate

Sugar maple leaves were collected after abscission from the TRT and REF catchments, dried at 60 °C and stored in plastic bags. Sugar maple foliage from the TRT catchment had higher N and P concentration than foliage from the REF catchment (Table 2). Leaves were mechanically ground and allowed to leach in deionised water for 2 weeks at 2 °C in 1-L HDPE bottles to yield DOM_{TRT} and DOM_{REF} leaf leachates. Leachates were decanted, centrifuged and filtered through Whatman GF/F (0.7 μm pore size) filters. Leachates were then sterilised by Tyndallisation by heating to 70 °C, allowing them to cool, and held at 25 °C for 24 h in three successive cycles (Kaplan *et al.*, 2008) and stored for <2 weeks at 2 °C until use. C, N and P concentrations of the resulting leachates are reported in Table 2.

Bottle assay sampling

All 64 bottles were sampled non-destructively at the time of incubation preparation (T_0) and after 2 (T_2), 10 (T_{10}) and 30 days (T_{30}). On each date, 60 mL of solution was taken from the bottles and filtered (0.7 μm GF/F) into HDPE bottles and held at −20 °C prior to the determination of NO_3-N, NH_4-N, SRP and DOC concentrations. Additional 10-mL filtered samples, to characterise DOM using fluorescence excitation–emission spectroscopy, were collected and held for up to 48 h at 2 °C in the dark prior to analysis. At T_{30}, an unfiltered aliquot was collected and stored at −60 °C prior to the analysis of microbial enzyme activity.

Field experiment

In May 2011, we measured the uptake of DOC derived from TRT leaves in both TRT and REF streams under ambient and elevated inorganic N and P. The DOM used in the field experiment was produced from a mixture of leaves from sugar maple and American beech, the two dominant tree species near the BBWM streams, collected after abscission from the TRT catchment. To make the leachate, leaves were dried, shredded and soaked in deionised water for 48 h at 40 °C. The slurry was sieved through a 250-μm mesh to removed particulate matter and refrigerated until it was used the next day. The chemical characteristics of the DOM leachate used in the field experiment are reported in Table 2. During the field leachate addition, discharge was 4 L s^{-1} in the TRT stream and 5 L s^{-1} in the REF stream. Under initial ambient conditions, NO_3-N was high in the TRT stream compared to REF, but SRP concentrations were similar (Table 3).

DOC uptake was assessed using a pulse release of the leachate and a conservative tracer, following Fellman *et al.* (2009b). In each release, a slug of 4 L of leachate and dissolved NaCl (100 g) was added to the stream *c.* 10 m upstream of the first sampling transect. Study reaches of 60 m were established in each stream (travel time *c.* 15 min) with six sampling locations equally distributed along their length. At each location, water samples (n = 3)

Table 2 Chemical characteristics of leaves and DOM leachates used in the laboratory and field experiments

	Source	Carbon	Nitrogen	Phosphorus	C : N: P
Leaves (mg kg^{-1})	REF sugar maple	473 570	5210	160	7893 : 74 : 1
	TRT sugar maple	471 170	7013	192	6544 : 84 : 1
Leachates (mg L^{-1})	REF sugar maple	3 578	22	1.6	5960 : 31 : 1
	TRT sugar maple	4 816	32	2.0	6217 : 36 : 1
	TRT sugar maple-beech mix	2 151	19	2.6	2238 : 17 : 1

Leaf C, N and P are total in mg kg^{-1} and leachate C is DOC, N and P are total dissolved, all in mg L^{-1}. C : N: P is molar ratio.

Table 3 Uptake metrics for DOC and mean reach nutrient concentrations during the whole-stream additions of leaf leachate. REF and TRT are the streams in the reference and treatment catchments, respectively. S_w = uptake length, V_f = uptake velocity and U = uptake rate

Stream	Treatment	S_w (SE) (m)	V_f (mm s^{-1})	U (μg m^{-2} s^{-1})	DOC (mg L^{-1})	NO_3-N (μg L^{-1})	SRP (μg L^{-1})	DOC_{bgd} (mg L^{-1})
TRT	Amb.	28 (3.08)	0.098	0.61	6.23	270	<1	0.170
	+NP	23 (2.01)	0.116	0.71	6.07	800	19	
REF	Amb.	31 (3.41)	0.118	0.79	6.70	<1	<1	0.522
	+NP	24 (2.23)	0.156	1.03	6.59	438	38	

Mean reach DOC, NO_3-N and SRP were calculated as the geometric mean of all samples taken at the peak of the DOM pulse along the reach. DOC_{bgd} is the mean reach DOC prior to leachate addition. S_w standard error is based on error estimate of k_L.

were collected and filtered (0.7 μm GF/F) before addition and again at the peak of the slug pulse determined by monitoring conductivity as a proxy for Cl^- concentration. DOC uptake was measured simultaneously in each stream reach, first under ambient N and P. After completing the initial DOC uptake assay at ambient N and P, we manipulated nutrient availability in each stream by adding a solution of $NaNO_3$, KH_2PO_4 and NaCl at a constant rate designed to increase concentrations by 500 μg L^{-1} NO_3-N and 100 μg L^{-1} P. After the conservative tracer had reached a steady state (*c.* 2 h), as indicated by specific conductance monitored at the downstream end of the study reach, we repeated the DOC uptake assay as described above in each stream. All water samples were kept on ice, returned to the laboratory and refrigerated until analysis for DOC, NO_3-N, SRP, Cl^- and fluorescence within 48 h. DOC uptake lengths (S_w) were calculated as the inverse of the longitudinal loss rate constant (k_L). Uptake velocity (V_f) was calculated as $V_f = uz/S_w$ and areal uptake U as $U = V_f\ C$ (where u is mean reach velocity, z is mean reach depth, and C is the ambient nutrient concentration; Webster & Valett, 2006).

Sample processing

Water samples were analysed for Cl^-, NO_3-N and SRP concentrations using a Lachat Quikchem 8500 analyzer (Hach company, Loveland, CO, U.S.A.) using methods E10-117-07-1-C, E10-107-04-1-B and E10-115-01-1-B, respectively. NH_4-N was analysed using the fluorometric technique (Holmes *et al*., 1999; Taylor *et al*., 2007). Method detection limits were 1 μg N L^{-1}, 2 μg N L^{-1} and 2 μg P L^{-1} for NO_3-N, NH_4-N and SRP, respectively. DOC concentrations were measured using a Shimadzu TOC analyzer (Shimadzu scientific instrument, Columbia, MD, U.S.A.). DOC consumption rates in the laboratory experiment were calculated as the slope of ln-transformed DOC concentration versus experiment day. Total C and N concentrations of leaves used to make leachates were determined by combustion using a LECO CN-2000 analyzer (LECO corporation, St-Joseph, MI, U.S.A.), and P concentration was determined by ICP-AES (IRIS-1000; Thermo Fisher Scientific, Waltham, MA, U.S.A.) after ashing and dissolving ash in HCl. Total N and total P of leachates were determined using the dual digestion technique with persulphate (APHA, 2005) before analysis on the Lachat Quickchem described above.

Fluorescence scans of each sample were conducted to characterise DOM composition. Scans were made using a Hitachi F-4500 spectrofluorometer (Hitachi, Tokyo, Japan) with the excitation range set from 240 to 400 nm and the emission range set from 300 to 500 nm, in 3-nm increments. Instrumental parameters were as follows: slits, 5 nm; response time, 8 s; and scan speed, 2400 nm min^{-1}. Excitation–emission matrices (EEM) resulting from the fluorescence scans were examined for peak maxima and used in parallel factor analysis (PARAFAC). PARAFAC is a multiway statistical method to decompose a suite of EEM landscapes into scores and loading vectors that estimate relative concentrations of the chemical components of the DOM pool and their excitation and emission spectra (Ohno & Bro, 2006; Stedmon & Bro, 2008). Raman and Rayleigh scatter effects were minimised according to methods described by Stedmon & Bro (2008). EEMs were not corrected for instrumental bias or inner filter effects because we were primarily interested in relative differences in the same samples over time. The PARAFAC model was constructed using all samples (n = 340) from the laboratory and field experiments. We focussed our analysis of the effects of stream and DOM source, and nutrient enrichment on the PARAFAC components whose relative contribution to fluorescence declined over the course of the experiment, suggesting they were preferentially consumed. The change in the proportion of a component over the course of the experiment is reported as Δ component X (proportion of component X at T_0 – proportion of component X at T_{30}).

The activities of three microbial enzymes associated with C (β-1,4-glucosidase: GLUC), N (leucine-aminopeptidase: LAMP) and P (phosphatase: PHOS) acquisition from the organic matter were assayed. Enzyme activity was assessed using fluorescent-labelled sub-strates (4-MUB-β-glucoside, 4-MUB-phosphate and L-leucine-7-amido-4-methylcoumarin) in conjunction with a microplate fluorometer (Fluoroskan Ascent FL; Thermoelectron, Waltham, MA, USA). Samples (200 μL) were added to 96-well microplates containing 50 μL of a 200 μM substrate solution. Fluorescence was measured at 25 °C. Analyses included reference standards and controls for particle quench, sample and substrate fluorescence (Findlay *et al*., 2003; Simon, Simon & Benfield, 2009).

Statistical analysis

Three-way analysis of variance (ANOVA) was used to evaluate the effect of stream source, DOM source and nutrient treatment on the response variables in the laboratory experiment. When interactions between stream source, DOM source and nutrient amendment occurred, stream sources were examined individually using two-factor (DOM source and nutrient amendment) ANOVA. *Post hoc* comparisons were made using Tukey's honestly

significant difference. For the field experiment, uptake lengths of DOC under ambient and elevated N and P for each stream were compared by testing for differences in ln-normalised slopes of DOC : Cl versus distance (i.e. k_L) using analysis of covariance (ANCOVA) with a dummy variable technique (Kleinbaum, Kuper & Muller, 1988). The results were considered statistically significant at $\alpha = 0.05$. All statistical analyses were performed using SYSTAT 12 software (SYSTAT software, Chicago, IL, U.S.A.). Parallel factor (PARAFAC) analysis of fluorescence was conducted using the Matlab PLS tool box (Mathworks, Natick, MA, U.S.A.).

Results

Laboratory experiment

Initial mean DOC concentration for all treatments was 9.4 mg C L^{-1} (SE = 0.38, $n = 64$) at T_0 and DOC declined nonlinearly over the course of the experiment in all treatment combinations. On average, 31% of the amended DOC was consumed over the course of the experiment. The effect of the inorganic nutrient treatment on mean DOC consumption rates was influenced by stream and DOM source (Table S1). In REF, DOM_{TRT} was consumed at an 18% faster rate than DOM_{REF} across nutrient treatments (Table S2; Fig. 1). The addition of P alone had no effect on DOC consumption rate, but the addition of N increased DOC consumption by 54% ($P < 0.001$; Fig. 1). In REF and TRT, simultaneous addition of N and P more than doubled the rate of DOC consumption of both DOM sources ($P < 0.001$; Fig. 1). In TRT, DOM_{TRT} was consumed at a 10% faster rate than DOM_{REF} across nutrient treatments, but this difference was not statistically significant (Table S2; Fig. 1). However, DOM source affected the magnitude of the N + P effect (Table S2; Fig. 1) with a 3.2-fold increase in the consumption of DOC from DOM_{TRT} compared to a 2.7-fold increase with DOM_{REF}.

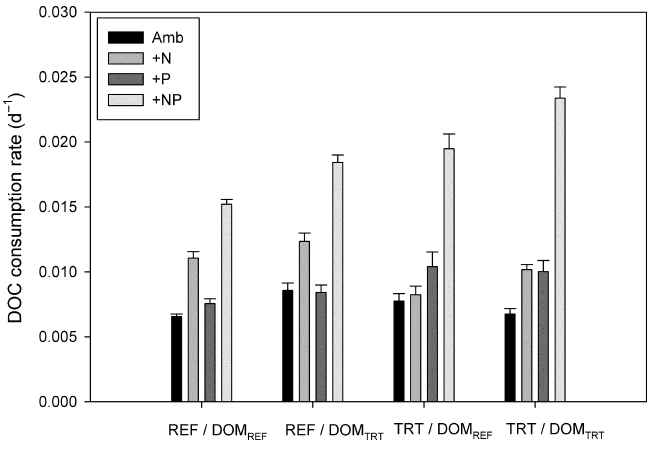

Fig. 1 Response of DOC consumption rate (slope of ln-transformed DOC concentration versus time) to stream, DOM source and inorganic nutrient amendments in the laboratory assays. Data are means ($n = 4$) + standard error. Stream and DOM source treatment combinations are indicated on the X axis, while inorganic nutrient treatments are represented by the bar shading.

PARAFAC analysis revealed four distinct components within the DOM pool. Three components had humic-like fluorescence spectra (components 1 (245/320 : 440 nm excitation/emission), 2 (370/275 : 460) and 4 (320/<250 : 450)) (Fellman *et al.*, 2009a; Yamashita *et al.*, 2010). One component (component 3 (275/345)) had a fluorescence signature typical of protein-like compounds (e.g. component 8 in Fellman *et al.*, 2009a). Over the 30-day incubation, the contributions of components 1 and 2 increased, while the contributions of components 3 and 4 declined across all treatment combinations (Fig. 2a). At the start of the experiment, the labile components were slightly more strongly represented in DOM_{TRT} with a combined mean proportion of components 3 and 4 of 52% (SE 1.2) compared to 46% (SE 0.7) for DOM_{REF}.

While the contributions of components 3 and 4 declined over time universally, they did so by different magnitudes across treatments. For the protein-like component 3, there was a significant 3-factor interaction effect ($P = 0.014$) so streams were analysed separately. In REF, the decline in component 3 was only influenced by nutrient treatment (Table S2). Without nutrient amendment, mean Δ component 3 was −0.1 and was reduced by 30% to −0.07 when N ($P = 0.010$) and N + P ($P = 0.012$) were added, whereas P addition had no effect ($P = 0.881$; Fig. 2b). In TRT, DOM source affected the response of Δ component 3 to the nutrient treatment (Table S2). For DOM_{REF}, P amendment reduced Δ component 3 by about 50% relative to ambient ($P = 0.012$) and N + P addition drastically reduced Δ component 3 from −0.07 to −0.001 ($P < 0.001$). For DOM_{TRT}, only N + P reduced Δ component 3 (−0.06 to −0.008; $P = 0.001$; Fig. 2b).

Unlike component 3, Δ component 4 was increased rather than reduced by nutrient amendment (Fig. 2c). The effect of nutrient amendment on Δ component 4 was influenced by the stream and DOM source (Table S1). In REF, the decline in component 4 was influenced by nutrient addition, but not DOM source (Table S2). The addition of P had no effect on Δ component 4 ($P = 0.38$; Fig. 2c). In contrast, the addition of N ($P = 0.02$) and N + P ($P < 0.001$) doubled the decline in component 4 (Fig. 2c). In TRT, the effect of nutrient addition depended on the DOM source (Table S2). For DOM_{REF}, P ($P = 0.052$)

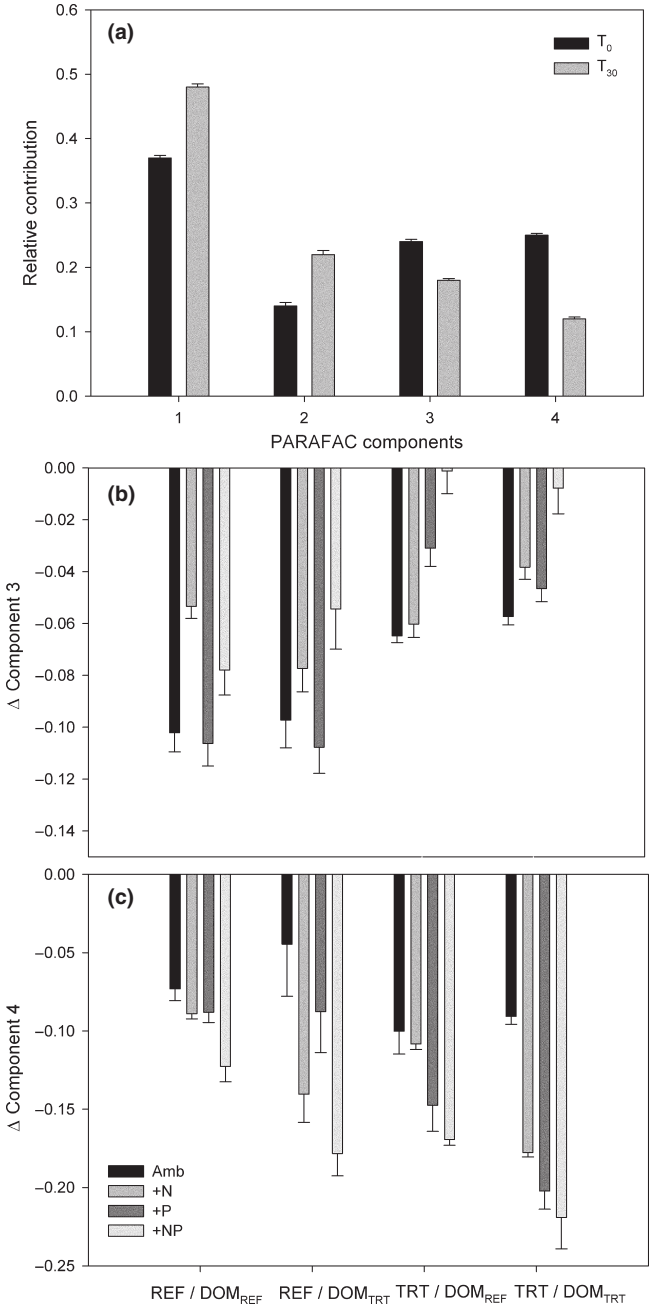

Fig. 2 (a) Mean relative contribution of parallel factor analysis (PARAFAC) components 1–4 during the laboratory experiment at T_0 (black bars) and T_{30} (grey bars). Components 1, 2 and 4 are humic-like, and component 3 is protein-like. (b) Change in component 3 during the bottle incubation assay (T_0 to T_{30}). (c) Change in component 4 during the bottle incubation assay (T_0–T_{30}). Error bars represent SE [in (a) n = 64, in (b) n = 4]. Stream and DOM source treatment combinations are indicated on the X axis, while inorganic nutrient treatments are represented by the bar shading.

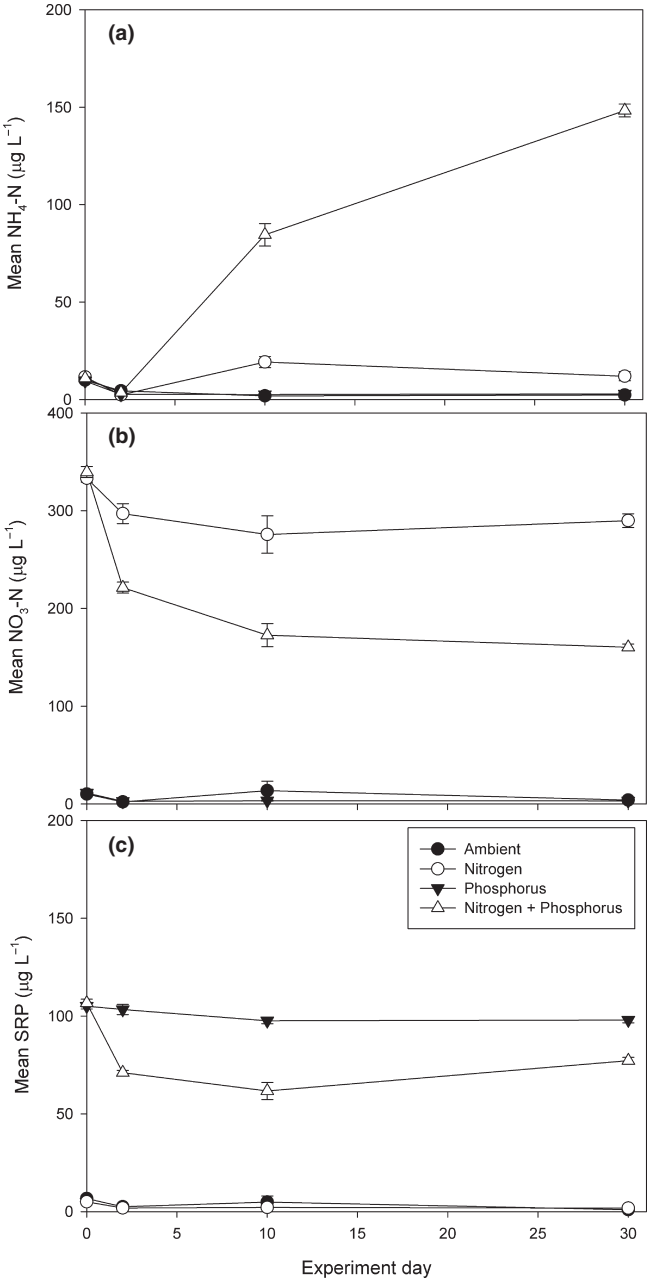

Fig. 3 Change in mean nutrient concentration during the laboratory experiment according to nutrient treatment for (a) NH_4-N, (b) NO_3-N and (c) SRP. Error bars represent ±SE (n = 16).

and N + P (P = 0.005; Fig. 2c) amendment increased Δ component 4 by about 50% (Fig. 2c). For DOM_{TRT}, N, P and N + P amendment approximately doubled Δ component 4 ($P \leq 0.001$; Fig. 2c).

Stream source influenced the effect of nutrient amendment on the change in NH_4-N and NO_3-N concentration over time (Table S1). NO_3-N amendment stimulated NH_4-N production and NO_3-N consumption over time only in REF ($P \leq 0.016$), while N + P amendment had a similar but larger effect for both stream sources ($P < 0.001$). Combined N and P amendment led to a large increase in NH_4-N (mean = 137 μg L^{-1}; Fig. 3a). When P was added in conjunction with N, about half of the amended NO_3-N

was consumed (Fig. 3b). The amount of NH_4-N produced in the N and N + P treatments equalled 62% of the NO_3-N decline in the N and N + P enriched bottles (Fig. 3). The change in SRP concentration over time was strictly affected by the nutrient treatment (Table S1) with N + P amendment resulting in a 25% consumption of the amended P (Fig. 3c).

The influence of any single level of stream, DOM source and/or nutrient amendment on all microbial enzyme activities depended on levels of the other factors (three-way factor interaction, $P < 0.001$), so streams were examined separately with DOM and nutrient amendment as main factors. For both streams, PHOS activity responded to nutrient amendment, but not DOM source (Table S2). The addition of N increased PHOS activity up to two-fold relative to controls for both streams, while the addition of P alone or in combination with N reduced PHOS activity by half in TRT, but had no effect in REF (Fig. 4a). GLUC activity in TRT was unresponsive to DOM source and nutrient amendment (Table S2; Fig. 4b). In REF, GLUC responded to DOM source and nutrient amendment, but there was a significant interaction between DOM source and nutrient amendment (Table S2). For both types of DOM, simultaneous amendment with N and P increased GLUC activity (Fig. 4b), but the effect was twice as large for DOM_{TRT} than that from DOM_{REF} (data not shown). LAMP activity was unresponsive to DOM source and nutrient amendment in REF (Table S2). For TRT, LAMP response to nutrient amendment depended on DOM source (Table S2). There were no effects of nutrient amendment with DOM_{REF}, but LAMP activity was increased slightly with the addition of N and DOM_{TRT} (Table S2, data not shown).

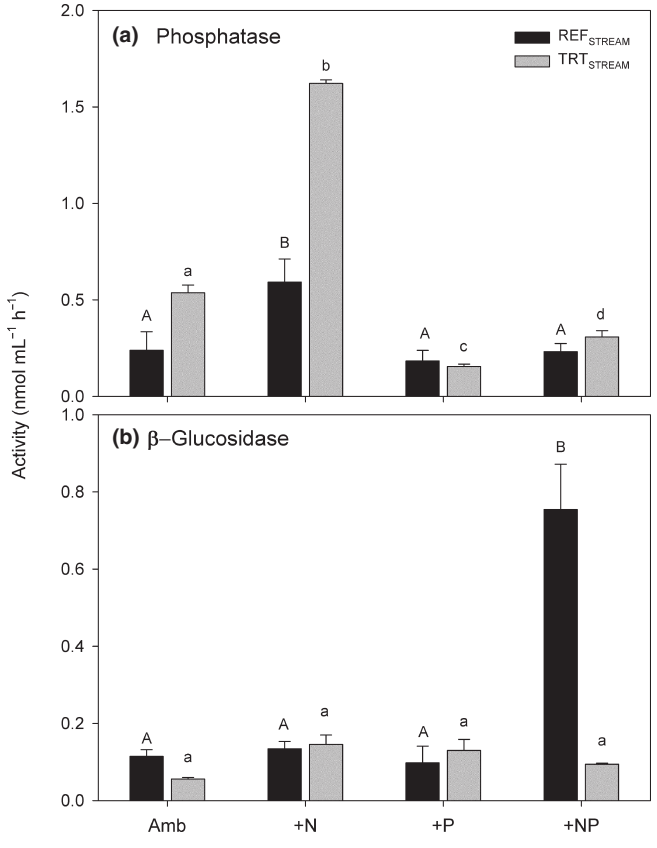

Fig. 4 Influence of inorganic nutrient amendment on activity of (a) phosphatase and (b) β-glucosidase at T_{30} of the laboratory experiment. Data are for REF (black, capital letters) and TRT (grey, lowercase letters) dosed with DOM_{REF} only. Bars with the same letters are not statistically different (Tukey's HSD, $P > 0.05$). Error bars represent SE ($n = 4$).

Field experiment

DOC uptake was rapid and similar in REF stream and TRT stream under ambient N and P (Table 3). The addition of DOM to the TRT stream caused a large decrease (*c.* 30%) in ambient reach mean NO_3-N concentration. We could not observe a similar effect on NO_3-N in the REF stream or on SRP in either stream because ambient concentrations of those solutes were so close to the detection limit that any decrease was not measureable. Under experimentally elevated NO_3-N and SRP concentrations, DOC uptake length declined by 23% compared to ambient in the REF stream ($P = 0.031$). While calculated S_w under elevated N and P was shorter than under ambient (18%) in the TRT stream, the difference was not statistically significant ($P = 0.088$) (Table 3). V_f and U were greater under elevated N and P compared to ambient conditions in both streams (Table 3). The same PARAFAC components that declined in the laboratory experiment also declined with distance downstream in the stream experiment under ambient and elevated inorganic N and P (data not shown).

Discussion

Microbial capacity to degrade and use DOM in freshwater systems has been linked to both DOM nutrient content (Kroer, 1993) and the availability of inorganic nutrients in water (Bernhardt & Likens, 2002; Brookshire *et al.*, 2005), particularly in regard to N. In the case of whole-catchment N enrichment, both pathways of altered nutrient availability are likely to be manifest because both organic matter and stream water nutrient concentrations are altered. Our results suggest that both DOM and inorganic nutrient stoichiometry affected the microbial consumption of DOM; however, the effects of altered DOM nutrient

content were relatively subtle. Furthermore, stream source modulated the response to DOM source and inorganic nutrient enrichment in several instances, indicating that the composition of the microbial community may influence the relative importance of these factors. Although DOM stoichiometry, inorganic nutrient availability and microbe/water source can all affect DOM bioavailability, we found that inorganic N and P co-limitation appears to be the dominant driver of reach-scale processing of leaf leachate DOM.

It is important to note that we addressed only the bioavailability of DOM that can be directly extracted from senesced leaves. Direct leaching of DOM from leaves can be a large source of DOM to streams (Meyer, Wallace & Eggert, 1998), but it is seasonal and not the sole source of DOM. In addition, leaf leachate DOM can be substantially more labile than throughfall, soil and stream DOM (Qualls & Haines, 1992). As reported in previous studies (Qualls & Haines, 1992; Strauss & Lamberti, 2002; Sobczak, Findlay & Dye, 2003; Bernhardt & McDowell, 2008), DOC in our leaf leachate was readily consumed in the laboratory and field experiments, suggesting much of the DOM consumed was quite labile. However, the decline in the humic-like component in the laboratory and field indicates that more refractory pools of DOM were also consumed. Consequences of N deposition for the bioavailability of ambient soil and stream DOM may differ from our findings and merit further research.

The long-term N enrichment and acidification at Bear Brook Watershed in Maine (BBWM) has enhanced N and P concentration in several tree species (Elvir *et al*., 2005), including the sugar maple leaves used in our study. The altered leaf chemistry resulted in lower C : N, but not C : P, in the DOM extracted from leaves. Such higher N content in DOM is generally considered to lead to higher bioavailability to microbes (Kroer, 1993). Consistent with this, the N-rich DOM_{TRT} was more readily consumed than the DOM_{REF} in the laboratory experiment. However, the difference in DOC consumption between DOM types (14%) was much smaller than what could be induced by the enhancement of inorganic N and P in water (≥2-fold). Likewise, DOM source effects on Δ components, nutrient concentrations in water and microbial enzyme activity were either absent or smaller than the effects of inorganic N and P amendment. The difference in effect size between DOM source and inorganic nutrient amendment could be due to the difference in the magnitude of increase in total N and P caused by these two treatments. For example, DOM_{TRT} total N concentration is 30% greater than DOM_{REF}, while the inorganic N amendment increased N concentration in the mesocosms 17-fold, on average. However, the difference in N and P content of the two DOM types reflects real system responses to the catchment-scale N enrichment. Similarly, the magnitude of inorganic N enrichment in our laboratory experiment mirrored the amount of NO_3-N enrichment typical of the stream in the TRT catchment (Navrátil *et al*., 2010). Therefore, the stronger effect of the inorganic nutrient addition compared to the difference in DOM source suggests that the NO_3-N enrichment resulting from the catchment-scale N addition may play a stronger role in DOM processing than do changes to DOM chemistry.

We expected that the N enrichment of DOM would alleviate N and exacerbate P limitation of microbial use of DOM. Such shifting nutrient limitation with altered organic matter N and P content has been shown for microbes on particulate detritus (Güsewell & Gessner, 2009). However, we found that stream source had a greater effect than DOM source on DOC consumption when N and P were added individually. Our laboratory experiment was conducted when absolute differences in NO_3-N between the REF and TRT streams were at a seasonal minimum, so we maximised the likelihood of observing any effects of altered DOM stoichiometry. This suggests that even small changes in stream water NO_3-N availability (REF = 4, TRT = 17 μg L^{-1}) may override any potential effect of altered DOM nutrient content. Nonetheless, when N and P were added simultaneously, DOM_{TRT} was consumed more readily than DOM_{REF}, suggesting that DOM nutrient supply may be important only when inorganic nutrient limitation is alleviated.

The results from our whole-stream additions also suggest a limited influence of DOM composition on uptake capacity in our streams. In general, the uptake velocity (V_f) of DOC in the streams at BBWM was fast compared to other studies. For example, values for DOC V_f in the BBWM streams (0.098–0.118 mm s^{-1}) were comparable to V_f of acetate (0.000–0.463 mm s^{-1}; Johnson & Tank, 2009; Johnson, Tank & Arango, 2009) and glucose V_f (0.008–0.122 mm s^{-1}; Newbold *et al*., 2006; Bechtold, 2010) and were faster than those measured for salmon carcass leachate (0.063 mm s^{-1}; Fellman *et al*., 2009b) and urea (0.028 mm s^{-1}; Brookshire *et al*., 2005) in other streams. Acetate, glucose, urea and salmon carcass leachate should all be rather labile DOM because of their molecular simplicity and/or high N content. The lack of difference in V_f between labile monomers and BBWM leaf leachate may be due to the considerable flexibility in the microbial capacity to use DOM (Findlay, Hickey & Quinn, 1997) and the relative lability of DOM directly extracted from the leaves compared to ambient DOM. It is likely that our uptake metrics represent mostly the highly labile

fraction of the DOM, although humic-like fractions also declined. In addition, abiotic adsorption may also account for some of the DOC retained. Nonetheless, the DOC V_f values in this study were faster than those reported for sugar maple leachate in two streams at Hubbard Brook (0.000–0.029 mm s^{-1}, Bernhardt & McDowell, 2008). This suggests that the leaf leachate used in this study was rather bioavailable and/or that demand for DOM in the BBWM streams is particularly high; both may contribute to the relatively subtle effect of DOM stoichiometry on DOC consumption observed in the bottle assay.

The effects of inorganic nutrient availability on DOC consumption that we observed are consistent with some studies (Zweifel, Norrman & Hagström, 1993), but not others (Sobczak *et al.*, 2003). Typically, C and N are thought to be strongly coupled, and many studies have addressed the ability of NO_3 to stimulate DOC use in streams (e.g. Bernhardt & Likens, 2002; Brookshire *et al.*, 2005). For example, Brookshire *et al.* (2005) found that experimental N enrichment of streams substantially increased the uptake of DOC. While amendment with N stimulated DOC consumption in the laboratory experiment, the effect was much weaker than the effects of combined N and P amendment. Our reach-scale additions of DOM also suggest that N alone was not an overriding regulator of microbial consumption of DOM. Our stream additions of DOM were conducted when NO_3-N concentration in the TRT stream (270 μg L^{-1}) was much higher than in the REF stream (<1 μg L^{-1}) and approaching the magnitude of N amendment in the laboratory experiment. Had N strongly limited DOM consumption, we should have seen higher ambient DOC uptake velocity in the TRT stream than in the REF stream. Instead, we saw higher uptake in the REF stream, suggesting higher NO_3-N alone did not result in higher DOC uptake capacity at the stream-reach scale.

Several lines of evidence from the laboratory experiment suggest inorganic N enrichment exacerbated P limitation, with consequences for DOC consumption. P limitation of microbial DOC use has been previously reported in marine systems (Kritzberg, Arrieta & Duarte, 2010) and wetlands (Wiegner & Seitzinger, 2004), but has not received much consideration in streams. Sole addition of N enhanced PHOS activity, consistent with increased microbial P limitation (Chróst & Siuda, 2003; Scott *et al.*, 2009), but also an increased capacity to acquire P from DOM. The higher DOC consumption with N addition suggests that enhanced PHOS activity facilitated C acquisition by providing P, but not to the extent that could be induced by the inorganic P addition. Notably, the amount of organic P added via the leachates was much lower than what was added in the inorganic +P treatment. This suggests that as inorganic N availability rises, its influence on DOC degradation ultimately will be constrained by the availability of organic and inorganic P for microbes.

The importance of microbial enzyme allocation to C, N and P acquisition was evident in our bottle assays. The addition of inorganic P reduced PHOS activity and stimulated NO_3-N uptake, yet it only increased DOC consumption when NO_3-N was readily available. Combined additions of N and P reduced PHOS activity, increased GLUC activity and yielded enhanced DOC consumption. Furthermore, the ratio of GLUC to PHOS activity decreased with inorganic N addition and increased with inorganic P addition, indicating that C acquisition is closely linked to inorganic N and P availability. These patterns are consistent with models of shifting microbial enzyme allocation with changing resource supply (Sinsabaugh & Moorhead, 1994). The coherent shifts in DOC, inorganic N and P pools and microbial enzyme activity observed in the bottle incubation indicate tight coupling of C, N and P by microbes in these systems.

These linkages between C, N and P also extend to shifts in DOM composition. Across all combinations of DOM types and nutrient amendment, the same PARAFAC components declined, but at different magnitudes. Notably, the addition of inorganic nutrients preferentially enhanced the consumption of a humic-like DOM component and decreased the consumption of the protein-like DOM component. This suggests that nutrient enrichment allows microbes to use more recalcitrant DOM pools such as humic components, which may be unavailable under ambient nutrient concentrations. Accordingly, changes in stream water inorganic nutrient availability may alter the composition of DOM due to enhanced microbial consumption of humic DOM under these conditions. Lutz *et al.* (2012) found that a stream-reach N addition stimulated the consumption of an autochthonous DOM component (320/250 : 420 nm), but did not affect a protein-like DOM component (270 : 355 nm). Therefore, the effect of inorganic nutrients on DOM composition may vary among streams, depending on ambient DOM, nutrient availability and metabolism.

DOC concentration and export in many eastern North American and European rivers have been increasing over the past several decades (Worrall *et al.*, 2004; Findlay, 2005). In some cases, there has also been a decline in net consumption of DOC by biota and both may be linked to increased N deposition (Findlay, 2005). Our results suggest that P availability may play a role in dictating microbial degradation of DOC in freshwater systems and

this perspective may need to be added to conceptual models of DOC and N dynamics. At BBWM, acidification has enhanced mobilisation of aluminium from soils to streams where it precipitates and adsorbs P (Norton *et al*., 2006). This acidification-induced P sink has been implicated in reduced microbial decomposition of particulate organic matter in streams draining catchments subjected to chronic acidification (Simon *et al*., 2009). Our findings suggest that as catchments recover from acid deposition, high in-stream processing of DOC may significantly mitigate the export of terrestrial DOC; however due to tight C, N and P coupling, this may only occur if a sufficient supply of P is available to microbial consumers.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. *P* values for 3-ANOVA. Bold values are significant ($P < 0.05$).

Table S2. *P* values for 2-ANOVA. Bold values are significant ($P < 0.05$).

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