

## Stream nutrient enrichment has a greater effect on coarse than on fine benthic organic matter

Cynthia J. Tant<sup>1</sup> AND Amy D. Rosemond<sup>2</sup>

*Odum School of Ecology, University of Georgia, Athens, Georgia 30602 USA*

Matthew R. First<sup>3</sup>

*Woods Hole Oceanographic Institute, Woods Hole, Massachusetts 02543 USA*

**Abstract.** Nutrient enrichment affects bacteria and fungi associated with detritus, but little is known about how biota associated with different size fractions of organic matter respond to nutrients. Bacteria dominate on fine (<1 mm) and fungi dominate on coarse (>1 mm) fractions, which are used by different groups of detritivores. We measured the effect of experimental nutrient enrichment on fungal and bacterial biomass, microbial respiration, and detrital nutrient content on benthic fine particulate organic matter (FPOM) and coarse particulate organic matter (CPOM). We collected FPOM and CPOM from 1 reference and 1 enriched stream. CPOM substrates consisted of 2 litter types with differing initial C:nutrient ratios (*Acer rubrum* L. and *Rhododendron maximum* L.). Fungal and bacterial biomass, respiration, and detrital nutrient content changed with nutrient enrichment, and effects were greater on CPOM than on FPOM. Fungal biomass dominated on CPOM (~99% total microbial biomass), whereas bacterial biomass dominated on FPOM (~95% total microbial biomass). These contributions were unchanged by nutrient enrichment. Bacterial and fungal biomass increased more on CPOM than FPOM. Respiration increased more on CPOM (up to 300% increase) than FPOM (~50% increase), indicating important C-loss pathways from these resources. Microbial biomass and detrital nutrient content were positively related. Greater changes in nutrient content were observed on CPOM than on FPOM, and changes in detrital C:P were greater than changes in detrital C:N. Threshold elemental ratios analyses indicated that enrichment may reduce P limitation for shredders and exacerbate C limitation for collector-gatherers. Changes in CPOM-dominated pathways are critical in predicting shifts in detrital resource quality and C flow that may result from nutrient enrichment of detritus-based systems.

**Key words:** nitrogen, phosphorus, headwater stream, carbon, detritus, shredder, fungi, bacteria, aquatic, freshwater, Coweeta Hydrologic Laboratory, southern Appalachian Mountains.

Increased nutrient mobilization and associated increased bioavailability of N and P are global problems in freshwater ecosystems (Smith and Schindler 2009). Effects of nutrient enrichment on detrital resources have received much less treatment in the literature on heterotrophic than on autotrophic food webs. Predicted effects of nutrient enrichment on heterotrophic pathways include increased respiration of C inputs driven by organisms ranging from

heterotrophic microbes to higher-order consumers (Dodds 2007). However, little is known about how different types of organic C resources (e.g., small vs large size fractions) or different groups of heterotrophic microorganisms respond to nutrient enrichment.

In stream ecosystems, particulate organic C resources can be divided into 2 functional types: coarse and fine particles. Coarse detritus in the form of leaves and wood provides most of the energy and nutrients in many forested headwater stream food webs, but fine benthic organic matter, which results from breakdown and fragmentation of coarse fractions and other processes, also is a significant resource for consumers and dominates the basis of consumer production in larger rivers (Wotton 1994, Wallace et al. 1997, Rosi-Marshall and Wallace 2002). In general,

<sup>1</sup> Present address: Department of Biological Sciences, State University of New York at Oswego, 7060 Route 104, Oswego, New York 13126 USA. E-mail: cynthia.tant@oswego.edu

<sup>2</sup> E-mail addresses: rosemond@uga.edu

<sup>3</sup> firstmr@saic.com

detrital nutrient content increases as particle size decreases (Stelzer et al. 2003). Microbial assemblages composed of fungi and bacteria are important decomposers of these resources. The relative biomass of bacteria and fungi differs on different detrital size fractions. Fungi dominate on coarse particulate organic matter (CPOM; wood and leaves), and bacteria dominate on fine particulate organic matter (FPOM) (Findlay et al. 2002). Heterotrophic microorganisms growing attached to or pervading organic matter resources are important facilitators of changes in quantity (via respiration) or quality (by decreasing C:nutrient ratios) of organic matter that they colonize (Stelzer et al. 2003).

Detritus is a poor-quality resource under ambient nutrient concentrations even when colonized by microbes, but nutrient enrichment can cause dramatic changes in detrital quality (Robinson and Gessner 2000, Pascoal et al. 2005, Chung and Suberkropp 2009). However, this quality response may differ for small- and large-sized detrital particles as a result of fungal vs bacterial dominance or the chemical and structural characteristics of substrates (e.g., differences in initial C:nutrient ratios, lignin content). Specifically, relatively greater fungal vs bacterial response to increased nutrient supply (Suberkropp et al. 2010) would differentially affect the substrates on which they dominate (CPOM vs FPOM, respectively; Findlay et al. 2002). In addition, nutrient enrichment effects can be greater for substrates with relatively low endogenous nutrient content, as is the case for wood vs leaf litter (Stelzer et al. 2003, Gulis et al. 2004) and leaf litter of species that differ in initial C:nutrient content (Greenwood et al. 2007). Moreover, the magnitude of change in C:nutrient ratio with enrichment is greater for CPOM than for FPOM, presumably because of lower initial nutrient content of CPOM and potentially greater reliance of microorganisms on external sources of nutrients (Cross et al. 2005). Investigators have assumed that microbial responses drive these trends, but the relationship between fungal and bacterial biomass and substrate quality under enriched conditions has not been tested.

Nutrient stimulation of microorganisms also can increase availability of detrital C to decomposers and detritivores. Nutrients can stimulate respiration rates of heterotrophic microorganisms, but whether this stimulation is greater on bacterial-dominated FPOM than fungal-dominated CPOM is not known. In addition, CPOM and FPOM are used by different groups of detritivores. Shredders are the primary consumers of CPOM, and collector-gatherers feed primarily on FPOM. Thus, differential effects of nutrient enrichment on CPOM vs FPOM could have

contrasting effects on these 2 functional groups of consumers and could lead to divergent cascading effects on energy flow through alternative foodweb pathways.

We quantified and compared bacterial and fungal biomass, microbial activity (respiration), and detrital nutrient content on benthic CPOM (from deployed leaf litter) and benthic FPOM (from depositional areas) in an experimentally nutrient-enriched and a reference stream. We estimated consumer threshold elemental ratios (TERs) and compared changes in detrital nutrient content to estimated consumer requirements as a way to assess implications of changes in detrital nutrient content for consumers. We predicted that: 1) nutrient enrichment would stimulate fungal and bacterial biomass, respiration rates, and associated nutrient content on both substrates; 2) changes in nutrient content with enrichment would be greater on CPOM than FPOM because of initially lower nutrient content of CPOM relative to FPOM; and 3) nutrient content would change as a function of fungi on CPOM and bacteria on FPOM because of their relative dominance on the substrates. We provided both N and P above concentrations considered to be growth limiting to heterotrophic microbes and roughly at Redfield ratios (Rosemond et al. 2008). Thus, we tested for relative changes in substrate C:N vs C:P that would indicate differential uptake of N vs P by detritus-associated microorganisms.

## Methods

### *Nutrient enrichment*

We conducted our study within the framework of a long-term, nutrient-enrichment, paired watershed study at the Coweeta Hydrologic Laboratory in southwestern North Carolina, USA. Enrichment was continuous from July 2000 to July 2007 (Rosemond et al. 2008, 2010). Data presented here are from samples collected during years 5 and 6 of enrichment (December 2004–June 2006). The overall study design consisted of collections of CPOM and FPOM from 2 spring-fed 1<sup>st</sup>-order streams, a reference and a nutrient-enriched stream. The streams are ~200 m apart and drain adjacent catchments on a south-facing slope in a mixed-deciduous forest in the Blue Ridge physiographic province at the southernmost tip of the Appalachian Mountains (Swank and Crossley 1988). Both streams are bordered by a dense understory of *Rhododendron maximum* L. and share similar physicochemical characteristics. The nutrient-enriched stream was continuously enriched in proportion to flow along its entire reach (150 m) with a solution of

ammonium nitrate and potassium phosphate delivered by drippers situated every 10 m along the stream bed. Flow-proportional dosing was achieved with a metering pump upstream that responded to a signal from an Isco stage-height recorder (Teledyne Isco, Inc., Lincoln, Nebraska) at a downstream weir. Before treatment, the reference and nutrient-enriched streams had similar nutrient concentrations (reference: dissolved inorganic nitrogen [DIN] =  $22 \pm 8.5$   $\mu\text{g/L}$ , soluble reactive P [SRP] =  $6.8 \pm 3.0$   $\mu\text{g/L}$ ; nutrient-enriched: DIN =  $29.3 \pm 4.9$   $\mu\text{g/L}$ , SRP =  $9.5 \pm 2.3$   $\mu\text{g/L}$ ). During the enrichment period prior to and inclusive of this study (July 2000–July 2007), nutrient concentrations in the reference stream remained low (DIN =  $31.0 \pm 4$   $\mu\text{g/L}$ , SRP =  $8.0 \pm 1.3$   $\mu\text{g/L}$ ), whereas concentrations in the nutrient-enriched stream increased moderately (DIN =  $506.2 \pm 36.3$   $\mu\text{g/L}$ , SRP =  $80.0 \pm 5.6$   $\mu\text{g/L}$ ). Further details of the enrichment, water sampling, and analysis are described elsewhere (Gulis and Suberkropp 2003, Rosemond et al. 2008).

#### *Organic matter sample collection*

We deployed 2 leaf-litter types, red maple (*Acer rubrum* L.) and rhododendron (*Rhododendron maximum* L.), that differ in their rates of breakdown and initial C:nutrient ratios. We collected recently senesced leaves in October 2004 and allowed them to air dry for  $\geq 2$  wk. We placed red maple or rhododendron leaves (15 g) in coarse mesh bags (5-mm mesh) and deployed the litter bags in each stream on 14 December 2004. We retrieved leaf packs periodically from the streams, rinsed the contents, and removed leaf discs for measurement of microbial activity (respiration) and fungal and bacterial biomass. We retrieved replicate red maple leaf packs ( $n = 3$ ) after 7, 14, 21, and 28 d and rhododendron leaf packs after 7, 14, 28, 37, and 49 d. We retrieved incubated leaf material until 25 January 2005. The leaf material remaining in a retrieved pack was dried at  $60^\circ\text{C}$ , weighed, and combusted at  $500^\circ\text{C}$  to measure ash-free dry mass (AFDM) remaining.

Deployment of previously uncolonized substrate for analysis of nutrient effects on FPOM was not feasible. Therefore, we based our analysis on collections of standing material in reference and nutrient-enriched streams over multiple dates (20 August 2005–30 June 2006). Sampling in this manner ensured that FPOM sampled had been exposed to enrichment in the nutrient-enriched stream for an extended period. Depending on the type of analysis, we collected samples on 11 to 18 sampling dates from 20 August 2005 to 30 June 2006 every 2 wk for the first 2 mo, then monthly. On each sampling date, we

collected 3 to 5 replicate grab samples of FPOM from the top 5 cm of sediments by stirring material within a small stovepipe corer (10-cm diameter) and filtering  $\sim 400$  mL of the slurry through a 1-mm screen. We removed a 100-mL fraction from each sample for measurement of respiration in the field. We placed the remaining volume of sample in a cooler, returned it to the laboratory, and preserved it for measurement of fungal and bacterial biomass.

#### *Microbial respiration*

We measured CPOM-associated microbial respiration as  $\text{O}_2$  uptake ( $\text{mg O}_2 \text{ g}^{-1}$  leaf AFDM  $\text{h}^{-1}$ ). We placed 10 leaf discs from each leaf pack in 30 mL of stream water in respiration chambers for 30 min and measured  $\text{O}_2$  reduction over time with YSI 5100 dissolved  $\text{O}_2$  meters (Yellow Springs Instruments, Yellow Springs, Ohio). We used additional respiration chambers with only stream water as controls. We dried leaf discs at  $60^\circ\text{C}$ , weighed them, and combusted them at  $500^\circ\text{C}$  to measure AFDM. We measured respiration for each leaf type on each retrieval date until adequate leaf material was no longer available.

We measured FPOM-associated respiration as  $\text{O}_2$  uptake ( $\text{mg O}_2 \text{ g}^{-1}$  AFDM  $\text{h}^{-1}$ ). We placed 100-mL samples in 150-mL biological  $\text{O}_2$  demand (BOD) bottles and filled the bottles to the top with filtered stream water to avoid displacing sample volume when removing the cap. We incubated samples for 2 h. We measured initial and final  $\text{O}_2$  ( $\text{mg/L}$ ) with a YSI 5100 dissolved  $\text{O}_2$  meter and YSI 5010 BOD probe. After completing respiration measurements, we filtered each 100-mL sample through a  $0.7\text{-}\mu\text{m}$ -pore-size glass-fiber filter (GF/F), which we dried at  $60^\circ\text{C}$ , weighed, and combusted at  $500^\circ\text{C}$  to measure AFDM.

#### *Microbial biomass*

We estimated fungal biomass by measuring ergosterol content of leaf material and FPOM. We placed 10 leaf discs from each leaf pack in 5 mL of methanol. We filtered 25-mL subsamples of FPOM through a GF/F and placed the filter in 5 mL of methanol. We extracted ergosterol from detrital samples by refluxing in methanolic KOH, followed by separation and quantification of ergosterol concentrations with High Performance Liquid Chromatography (HPLC) with a Whatman Partisphere C18 column and ultraviolet detector set at 282 nm (VP series M, Shimadzu, Columbia, Maryland; Newell et al. 1988, Suberkropp and Weyers 1996). We converted ergosterol concentrations to fungal biomass assuming a conversion factor of  $5.5 \mu\text{g/mg}$  of mycelial dry mass (Gessner and Chauvet 1993).

We estimated bacterial biomass by image analysis with epifluorescence microscopy (Olympus BH-2 microscope and Qcolor 3 digital camera, Center Valley, Pennsylvania) following staining with SYBR Gold (Molecular Probes, Inc., Grand Island, New York; Noble and Fuhrman 1998). We preserved bacterial samples from FPOM (2.5-mL aliquots) in 5% buffered formalin (final concentration = 2.5%) and placed 10 leaf discs from each leaf pack in 5 mL of 2% formalin. We removed bacteria from FPOM and leaf substrates by sonicating for 1.5 min (Bransonic 150 probe sonicator; Branson, Danbury, Connecticut). During sonication, we placed samples on ice every 30 s to prevent excessive heating (Buesing and Gessner 2002). We then centrifuged samples at 800g for 1 min to separate bacterial cells from particulate matter to improve image analysis. We diluted FPOM samples 1:10, and filtered 1 mL of each sample through a 0.2- $\mu$ m-pore-size, 25-mm-diameter, black polycarbonate filter to capture bacteria. We stained the filters with SYBR Gold (supplied at 10,000 $\times$ , final concentration = 25 $\times$ ) as described in Noble and Fuhrman (1998) and Lisle and Priscu (2004). We captured images from 20 random fields from the filter surface at 1000 $\times$  magnification with an Olympus BH-2 microscope and an Olympus Qcolor 3 digital camera. For each filter, we captured 20 microscope images and analyzed them with the MatLab image processing toolbox (version 7.9; MathWorks, Natick, Massachusetts). We used cell counts and cell body dimensions (length and width) to calculate cell biovolume and, in turn, mean bacterial biomass concentration (First and Hollibaugh 2008).

#### *C, N, and P content*

We dried leaves from leaf packs and 25-mL subsamples of FPOM at 60°C and ground samples in a ball mill prior to analysis for C, N, and P content. We weighed ground material and analyzed for C and N content with a Carlo Erba NA 1500 CHN analyzer (Carlo Erba, Milan, Italy). We analyzed P content spectrophotometrically after acid digestion (APHA 1998). Nutrient ratios were expressed on a molar basis.

#### *Statistical analyses*

The ecosystem-level scale used here provided natural experimental conditions for the responses tested. However, analyses were not strictly replicated because only 1 reference and 1 enriched stream were used, violating assumptions of inferential statistics (Hurlbert 1984). Thus, nutrient effects based on replicate and spatially distributed samples within streams should be interpreted with caution.

We used a 2-way analysis of variance (ANOVA) to test for main and interactive effects of nutrient enrichment and time on fungal biomass, bacterial biomass, microbial respiration, C:N, and C:P. In this analysis, time was treated as an independent fixed effect. We used 2-way analysis of covariance (ANCOVA) to test for effects of nutrient enrichment, litter type, and microbial biomass on substrate nutrient content. Nutrient enrichment and litter type were treated as fixed effects, and measures of microbial biomass were used as cofactors. We used linear regressions to compare responses between treatments in cases where nutrient  $\times$  litter type effects were significant. We also ran 1-way ANCOVAs for CPOM and FPOM to test whether relationships between C:P and C:N changed because of nutrient enrichment. We  $\ln(x)$ -transformed data to meet assumptions of normality and homoscedasticity. All statistical analyses were performed using SAS (version 9.1; SAS Institute Inc., Cary, North Carolina).

#### *Estimation of threshold elemental ratios (TERs)*

We estimated TERs to evaluate changes in detrital resources relative to nutritional requirements of consumers with the following formula given by Frost et al. (2006):

$$TER_{C:nutrient} = (A_{nut}/GGE_C)(Q_C/Q_{nut})$$

where  $A_{nut}$  is the assimilation efficiency of the nutrient (nut) N or P,  $GGE_C$  is the gross growth efficiency of the consumer based on C ingested,  $Q_C/Q_{nut}$  is the proportion of C:nutrient in consumer dry mass. We set assimilation efficiency for N and P at 0.8 and gross growth efficiency for C at 0.2 based on values taken by Frost et al. (2006) from literature sources. We took shredder and collector-gatherer body C:P and C:N used in estimations of TERs from data collected from our study streams and published by Cross et al. (2003).

## **Results**

#### *Effects of nutrient enrichment on microbial biomass and activity*

Nutrient enrichment resulted in higher fungal and bacterial biomass and microbial respiration on CPOM of both litter types, but affected only microbial respiration and bacterial biomass on FPOM (Table 1, Appendices S1–S3; available online from: <http://dx.doi.org/10.1899/12-049.1.s1>). Percent change in fungal and bacterial biomass and respiration were greatest for rhododendron substrate and least for FPOM (Table 2).

TABLE 1. Results of 2-way analyses of variance testing for effect of nutrient addition (Nutrient) on fungal biomass, bacterial biomass, microbial respiration, and C:N and C:P values on benthic fine particulate organic matter (FPOM), red maple, and rhododendron substrates. Date is treated as a fixed effect, and a significant interaction indicates that nutrient effects varied through time. ns = not significant ( $p > 0.05$ ). Bold indicates significant effects.

Variable	Factor	FPOM			Maple			Rhododendron		
		df	F	p	df	F	p	df	F	p
Fungal biomass	Nutrient	1,40	0.70	ns	1,18	20.65	<b>&lt;0.001</b>	1,20	113.81	<b>&lt;0.001</b>
	Date	9,40	13.55	<b>&lt;0.001</b>	4,18	20.01	<b>&lt;0.001</b>	4,20	73.67	<b>&lt;0.001</b>
	Nutrient × date	9,40	0.68	ns	3,18	0.72	ns	4,20	9.25	<b>&lt;0.001</b>
Bacterial biomass	Nutrient	1,38	5.42	<b>0.025</b>	1,4	9.21	<b>0.039</b>	1,20	20.16	<b>&lt;0.001</b>
	Date	9,38	7.63	<b>&lt;0.001</b>	1,4	15.17	<b>0.018</b>	4,20	11.72	<b>&lt;0.001</b>
	Nutrient × date	9,38	2.51	<b>0.023</b>	1,4	2.27	ns	4,20	4.77	<b>0.007</b>
Microbial respiration	Nutrient	1,126	105.00	<b>&lt;0.001</b>	1,16	9.83	<b>0.006</b>	1,20	5.71	<b>0.027</b>
	Date	17,126	5.22	<b>&lt;0.001</b>	3,16	2.95	0.064	4,20	3.74	<b>0.020</b>
	Nutrient × date	17,126	1.94	<b>0.020</b>	3,16	7.37	<b>0.003</b>	4,20	6.58	<b>0.002</b>
C:N	Nutrient	1,66	0.03	ns	1,12	172.16	<b>&lt;0.001</b>	1,16	41.65	<b>&lt;0.001</b>
	Date	9,66	4.49	<b>&lt;0.001</b>	2,12	6.18	<b>0.014</b>	3,16	14.94	<b>&lt;0.001</b>
	Nutrient × date	9,66	0.75	ns	2,12	1.80	ns	3,16	9.12	<b>&lt;0.001</b>
C:P	Nutrient	1,46	1.01	ns	1,12	112.70	<b>&lt;0.001</b>	1,16	32.64	<b>&lt;0.001</b>
	Date	6,46	3.75	<b>0.004</b>	2,12	2.31	ns	3,16	2.18	ns
	Nutrient × date	6,46	1.32	ns	2,12	0.47	ns	3,16	1.81	ns

Bacterial biomass dominated on FPOM (95% of total microbial biomass), whereas fungal biomass dominated on CPOM (99% of total microbial biomass) (Table 2), and the relative contributions of bacteria vs fungi did not change with nutrient enrichment (CJT, unpublished data). These patterns in dominance suggest that responses to nutrient enrichment associated with CPOM occurred via fungal biomass and activity and those associated with FPOM occurred via bacterial biomass and activity, a conclusion that is supported by our data.

Respiration rates increased more with nutrient enrichment on CPOM substrates (303% on rhododendron) than on FPOM (52%) (Table 2). On average,

respiration rates per unit microbial biomass were roughly similar on FPOM and CPOM substrates, but microbial-C normalized rates tended to be slightly higher on FPOM with enrichment and slightly lower on CPOM with enrichment (Appendices S2–S3). Despite these C-normalized trends, respiration rates increased more with enrichment on CPOM than on FPOM largely because total microbial biomass was greater on CPOM than on FPOM.

#### Nutrient enrichment effects on substrate nutrient content

C:N and C:P on both CPOM litter types were lower in the nutrient-enriched than the reference stream, but

TABLE 2. Mean ( $\pm 1$  SE) reference values for 5 variables on benthic fine particulate organic matter, red maple, and rhododendron substrates and % change with enrichment. Percent change is based on the mean of all samples in the reference stream compared to the mean of all samples in the nutrient-enriched stream.

Variable	FPOM		Red maple		Rhododendron	
	Reference	% change	Reference	% change	Reference	% change
Fungal biomass (mg C/g AFDM)	0.41 $\pm$ 0.07	21	10.23 $\pm$ 2.38	139	1.98 $\pm$ 0.34	453
Bacterial biomass (mg C/g AFDM)	8.05 $\pm$ 0.81	31	0.06 $\pm$ 0.02	64	0.02 $\pm$ 0.01	552
Microbial respiration (mg O <sub>2</sub> g AFDM <sup>-1</sup> h <sup>-1</sup> )	0.10 $\pm$ 0.01	52	0.17 $\pm$ 0.08	122	0.04 $\pm$ 0.02	303
C:N	19 $\pm$ 0.57	3	77 $\pm$ 1.75	-36	141 $\pm$ 6.99	-28
C:P	168 $\pm$ 16	-17	5888 $\pm$ 273	-72	8513 $\pm$ 699	-54

TABLE 3. Results of 2-way analyses of covariance testing for effects on benthic coarse particulate organic matter nutrient content. Fungal or bacterial biomass was used as covariates and nutrient enrichment and litter type as fixed effects. Bold indicates significant effects.

Variable	Factor	C:N			C:P		
		df	F	p	df	F	p
Fungal biomass	Fungi	1,34	25.63	<b>&lt;0.001</b>	1,34	35.28	<b>&lt;0.001</b>
	Nutrients	1,34	16.28	<b>&lt;0.001</b>	1,34	82.70	<b>&lt;0.001</b>
	Leaf species	1,34	23.07	<b>&lt;0.001</b>	1,34	1751.49	<b>&lt;0.001</b>
	Nutrients × species	1,34	0.39	ns	1,34	47.75	<b>&lt;0.001</b>
Bacterial biomass	Bacteria	1,27	20.42	<b>&lt;0.001</b>	1,27	13.81	<b>&lt;0.001</b>
	Nutrients	1,27	9.94	<b>0.003</b>	1,27	35.10	<b>&lt;0.001</b>
	Leaf species	1,27	23.51	<b>&lt;0.001</b>	1,27	5.14	<b>0.032</b>
	Nutrients × species	1,27	0.01	ns	1,27	0.98	ns

enrichment did not affect C:N and C:P on FPOM (Tables 1, 2, Appendices S1–S3). Percent change in C:P was greater than % change in C:N with enrichment. Averaged across all samples, % change in C:P and C:N with enrichment was greater for red maple than rhododendron (Table 2), but maximum changes in C:P and C:N were greater for rhododendron than red maple (−415 and −120% for C:P and C:N, respectively, for rhododendron vs −330 and −86% for C:P and C:N, respectively, for red maple).

*The role of microbes in driving nutrient content*

Fungal biomass and CPOM C:N were significantly negatively related. Nutrients and leaf species affected CPOM C:N, but their effects were not interactive. C:N was lower on leaves with greater fungal biomass, but neither nutrient enrichment nor leaf species changed the nature of the relationship between fungal biomass and C:N (Table 3, Fig. 1A). For every 1% increase in fungal biomass, CPOM C:N decreased 0.23% (Fig. 1A). Fungal biomass and CPOM C:P also were significantly negatively related, and nutrient enrichment and leaf species affected CPOM C:P. However, these effects were interactive, indicating lower C:P for a given fungal biomass in the nutrient-enriched than in the reference stream (Table 3, Fig. 1B). Significant negative relationships between fungal biomass and C:P occurred only on nutrient-enriched red maple and rhododendron samples. These regressions indicated a 0.19% reduction in red-maple C:P and a 0.25% reduction in rhododendron C:P for each 1% increase in fungal biomass (Table 4, Fig. 1B).

Bacterial biomass and CPOM C:N and C:P also were negatively related (Table 3, Fig. 2A, B). For each 1% increase in bacterial biomass, C:N decreased 0.22% and C:P decreased 0.34% on CPOM substrates (Table 4, Fig. 2A, B). No significant relationships were found between microbial biomass and nutrient content on FPOM substrates.

*Relationship between C:N and C:P*

The relationships between C:N and C:P on detrital substrates differed between the nutrient-enriched and reference streams. C:N and C:P on CPOM were significantly positively related in both streams, but C:P on CPOM was lower for a given C:N in the nutrient-enriched than in the reference stream over the range of values observed ( $p < 0.001$ ; Fig. 3A). C:N and C:P also were significantly positively related on FPOM. C:P was significantly lower for a given value of C:N in the nutrient-enriched than in the reference stream ( $p < 0.001$ ; Fig. 3B), but this effect was smaller than for CPOM.

*TERs*

Estimation of TERs for C:N and C:P suggested that collector-gatherers were more C than N or P limited, whereas shredders were more N or P than C limited under reference conditions (Table 5). The greatest imbalances were between shredder TERs and C:P of their food resources. Comparisons of detrital nutrient content (FPOM for collector-gatherers, CPOM for shredders) to TERs under enriched conditions indicated that enrichment may reduce the severity of P limitation for shredders, but did not change N limitation. Increased body P content was found in a previous study in this stream (Cross et al. 2003), so calculated TERs indicated even greater demand for P under enriched than under reference conditions (lower  $TER_{C:P}$ ). Had we not included changes in body stoichiometry in our calculations, we would have estimated even greater reduction in C:P imbalances between shredders and food resources in the nutrient-enriched stream (differences of −1071 vs −2055). Slight reductions in C limitation of collectors in the nutrient-enriched stream were driven by reduced C demand estimated by TERs.

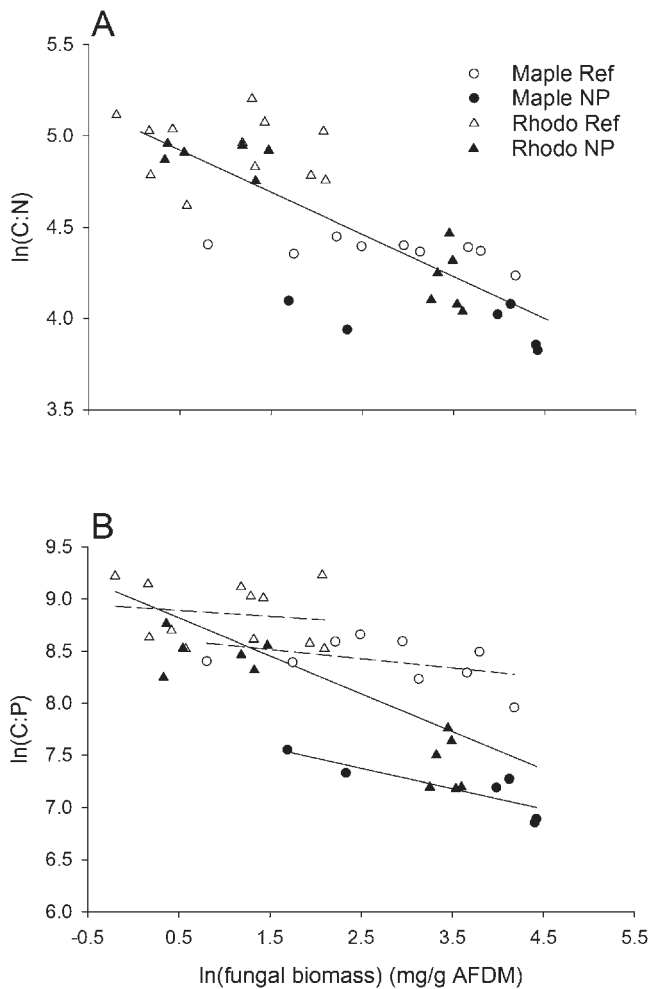


FIG. 1. Relationship between benthic coarse particulate organic matter fungal biomass and substrate C:N (A) and C:P (B) ratio. Solid regression lines indicate nutrient-enriched (NP), and dashed lines indicate reference (ref). AFDM = ash-free dry mass, rhodo = rhododendron.

## Discussion

### *Effects of nutrients on microbial colonization and respiration and implications*

Nutrient enrichment stimulated microbial biomass and activity on coarse and fine fractions of organic matter, but the overall response was much stronger on the coarse fraction. This larger response probably was a result of the dominance of fungi on CPOM and associated responses of increased microbial respiration and increased nutrient content. The largest responses on the 2 CPOM litter types tested were observed on the leaf species that had the lowest initial nutrient content (rhododendron), a result consistent with those of other studies (Gulis and Suberkropp 2003, Stelzer et al. 2003). Thus, if our results are transferable to other systems, nutrient enrichment is likely to result in a greater response on coarse than on fine substrates, and on substrates with relatively high C content or with microbial biomass dominated by fungi.

Our results are consistent with reported cross-system tendencies for greater bacterial biomass on FPOM and fungal biomass on CPOM (Table 2; Findlay et al. 2002). In our study, biomass of fungi and bacteria increased similarly in the nutrient-enriched stream, but the greatest responses of both bacteria and fungi were on CPOM. Presumably, both fungi and bacteria were able to exploit the bioavailable N and P in the water column in the nutrient-enriched stream, even when growing on the more recalcitrant rhododendron leaf litter. In fact, the greatest increases in bacterial and fungal biomass occurred on rhododendron substrates. Despite increases in both bacteria and fungi, fungal C dominated total microbial C on CPOM in reference and nutrient-enriched conditions (99.0% vs 98.8%). Thus, we think it likely that functional responses in nutrient content and respiration were caused by large changes in the mass and activity of fungi on CPOM.

TABLE 4. Results of linear regression analyses of microbial biomass vs benthic coarse particulate organic matter (CPOM) nutrient content. Analyses were run based on results of 2-way analyses of covariance testing for effects on benthic CPOM nutrient content. ns = not significant, SE = standard error, Ref = reference stream, NP = nutrient-enriched stream.

Model	<i>p</i>	<i>r</i> <sup>2</sup>	Slope	SE	Intercept
Fungal biomass vs C:N	<0.001	0.64	-0.23	0.03	5.04
Fungal biomass vs C:P					
Maple reference	ns	-	-0.09	0.07	8.65
Maple nutrient-enriched	0.031	0.66	-0.19	0.06	7.86
Rhododendron reference	ns	-	-0.03	0.07	8.87
Rhododendron nutrient-enriched	<0.001	0.84	-0.25	0.03	8.73
Bacterial biomass vs C:N	<0.001	0.47	-0.22	0.04	3.88
Bacterial biomass vs C:P	<0.001	0.38	-0.34	0.08	7.14

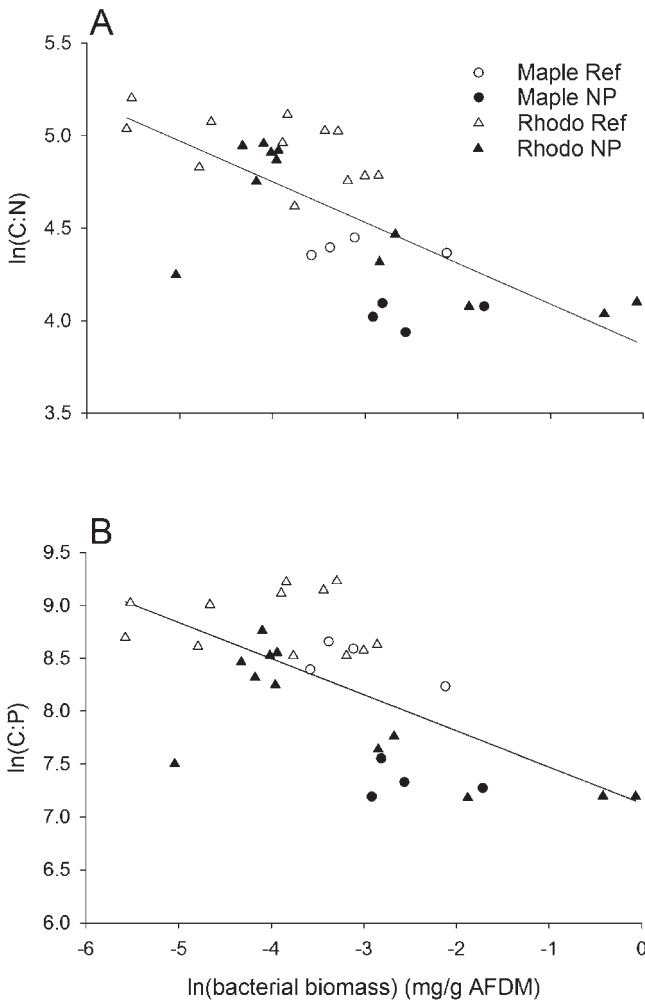


FIG. 2. Relationship between benthic coarse particulate organic matter bacterial biomass and substrate C:N (A) and C:P (B) ratio. AFDM = ash-free dry mass, rhodo = rhododendron.

Relationships between bacteria and nutrient content on CPOM may have been primarily a result of their associated response with fungi, which were driving these patterns.

Another potential explanation for smaller nutrient effects on FPOM than on CPOM substrates is the variable nature of fine particles. FPOM consists of material from multiple sources, including erosional inputs from allochthonous sources, aggregates of dissolved organic matter, fecal material, and particles released during maceration and breakdown of CPOM. These particles often have been significantly altered through microbial activity and macroinvertebrate consumption (Ward et al. 1994). Moreover, the age of FPOM in most stream systems is older and more varied than the age of CPOM, given that most CPOM, except for large pieces of wood, is converted

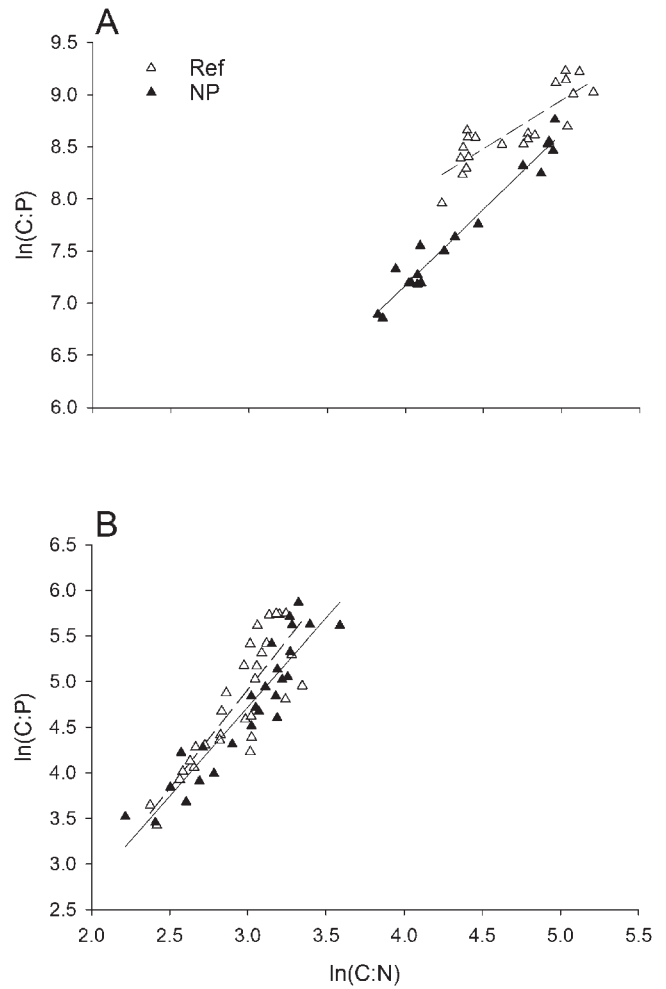


FIG. 3. Relationship between substrate C:N and C:P ratio of benthic coarse particulate organic matter (reference:  $p < 0.001$ ,  $r^2 = 0.72$ ,  $y = 0.93x + 4.29$ ; nutrient:  $p < 0.0001$ ,  $r^2 = 0.96$ ,  $y = 1.46x + 1.35$ ) (A) and benthic fine particulate organic matter (reference:  $p < 0.001$ ,  $r^2 = 0.77$ ,  $y = 2.32x - 2.00$ ; nutrient:  $p < 0.0001$ ,  $r^2 = 0.85$ ,  $y = 1.95x - 1.13$ ) (B). Solid regression lines indicate nutrient-enriched stream (NP). Dashed lines indicate reference stream (ref).

to FPOM within days to months. CPOM, such as leaf litter, is inherently high in C:nutrient content because plants translocate nutrients from leaves before senescence (Webster and Benfield 1986). Our sampling design resulted in collections of FPOM that were inherently more variable than CPOM (of unknown age and initial quality), and variability in the source and age of FPOM relative to CPOM could increase variation in response to nutrients. These differences in age and quality are likely to characterize CPOM and FPOM in other systems, albeit, not so dramatically. As a result of the controlled age of CPOM in this experiment vs the random sampling of in situ FPOM,



TABLE 5. C:nutrient threshold elemental ratios (TERs) and nutrient ratios of associated food resources for shredders and collector-gatherers in the reference and nutrient-enriched streams. Consumer TERs were estimated from body stoichiometry in the reference and nutrient-enriched streams taken from the study by Cross et al. (2003). Food resources for shredders were values from benthic coarse particulate organic matter taken from our study, and food resources for collectors were values from benthic fine particulate organic matter taken from our study. Elemental imbalance was assessed by subtracting the C:N or C:P of the associated food resource from the consumer's TER. Negative values indicate N or P limitation, and positive values indicate C limitation. Values approaching 0 indicate less limitation of any element. Estimated TERs are lower in the nutrient-enriched than in the reference stream because of changes in consumer body stoichiometry reported by Cross et al. (2003). Ref = reference stream, NP = nutrient-enriched stream.

Feeding group	Consumer TER		Food resource		Elemental imbalance	
	Ref	NP	Ref	NP	Ref	NP
Shredders						
C:N	27	26	57	55	-30	-29
C:P	1992	1008	4854	3063	-2862	-2055
Collectors						
C:N	26	24	19	20	+7	+4
C:P	1108	908	168	140	+940	+768

our findings may be skewed somewhat to facilitate detection of effects on less variable CPOM than on highly variable FPOM.

Respiration rates increased on both CPOM and FPOM in response to nutrient enrichment. Increased respiration rates indicate that fewer detrital resources will be available for downstream transport or storage (Cole et al. 2007, Aufdenkampe et al. 2011). Reduced C storage consequent to increased respiration rates also has been observed in terrestrial ecosystems (Mack et al. 2004). C budgets from the first 3 y of this long-term study revealed significant increases in downstream transport of FPOM and reductions in C storage with nutrient enrichment (Benstead et al. 2009). Nutrient enrichment increased respiration associated with both FPOM and CPOM, but the greatest effect was observed on the more recalcitrant leaf litter, a result also observed in a previous study in these streams (Gulis and Suberkropp 2003, Greenwood et al. 2007). Effects were greatest on substrates (in this case, rhododendron) that provide a more persistent, year-round food and structural resource for headwater-stream organisms. Thus, the response to chronic enrichment of aquatic ecosystems may differ not just among systems, but within different components of the ecosystems and are potentially predictable based on species traits.

#### *Effects of nutrients on microbial biomass and substrate nutrient content*

We found quantitative relationships between microbial biomass and detrital nutrient content on CPOM that will aid predictions of nutrient-enrichment effects on detrital food resources in aquatic

ecosystems. These data illustrate that microbial biomass can explain large amounts of variation in nutrient content with enrichment (64–84% for relationships with fungal biomass and 38–47% for relationships with bacterial biomass) on CPOM without changing nutrient content on FPOM. Relationships between fungal biomass and C:P differed between the nutrient-enriched and reference stream and between leaf species. The slope of the regression was greater for rhododendron (more recalcitrant, with high initial C:nutrient ratios) than maple (less recalcitrant, with lower initial C:nutrient ratios). Thus, as fungal biomass increased, C:P values of red maple and rhododendron in the nutrient-enriched stream converged (Fig. 1B). The stronger response of rhododendron than red maple to nutrient enrichment probably was caused by both structural and chemical differences. Rhododendron has a characteristically waxy cuticle, and freshly abscised leaves have high C:N and C:P (Webster and Benfield 1986). Thus, microbial colonization of this substrate is more dependent on nutrients available in the water column than on substrate-available nutrients. Previous investigators also have shown a stronger response to enrichment from microbes on lower-quality substrates (Gulis and Suberkropp 2003, Stelzer et al. 2003, Greenwood et al. 2007). The greater effects on C:P than C:N may be attributable to increased fungal colonization and to fungal capacity for storage of P vs N (V. Gulis [Coastal Carolina University], ADR, and D. Manning [University of Georgia], unpublished data). The relatively greater change in C:P than in C:N on fungal-dominated CPOM vs bacterial-dominated FPOM is consistent with this mechanism.

Consumer responses to enrichment hinge on changes in productivity and elemental changes in food resources. Our analysis of changes in detrital C:P and C:N suggested that when nutrients were supplied at roughly Redfield ratios, microbes immobilized more P relative to N. This differential immobilization results in large differences in the P content of detritus for a given amount of N content, particularly for CPOM substrates. For example, at a low C:N (e.g., 50) of CPOM, C:P in the reference stream would be predicted to be 2774. However, in the nutrient-enriched stream, at the same C:N, we would predict that C:P would be 1166, a value that might mean much lower P limitation for detrital consumers.

#### *Implications of altered detrital nutrient content for consumers*

Estimates of TERs highlight the potential for shifts in FPOM or CPOM foodweb pathways resulting from differential changes in food resources used by different functional groups. These effects also may interact with decreased seasonal availability of C that occurs with long-term enrichment (Suberkropp et al. 2010). The most severe imbalance between consumers and their food resources in our study were in the P content of CPOM to shredders, and the greatest change in detrital stoichiometry was for C:P on CPOM resources. Some functional groups (in this case shredders) may experience increases in production as a result of less nutrient limitation, whereas others, such as collector-gatherers, were more C than N or P limited based on their TERs. C imbalances were reduced somewhat with enrichment, but these shifts were observed only because we calculated TERs based on previous observations that C:P of consumers had declined in the nutrient-enriched stream (reducing their relative C demand). Otherwise, these organisms may have appeared vulnerable to greater C imbalances with increases in detrital nutrient content. These results suggest a potential mechanism to explain observed increases in both abundance and biomass of some CPOM consumers, such as the shredder *Pycnopsyche*, but a lack of response of other consumers under enriched conditions that resulted in increased detrital nutrient content (Davis et al. 2010). Elemental imbalances, although reduced for shredders, still persisted between consumers and their food resources in the enriched stream. Detritivore and heterotrophic microbe dynamics play a key role in C fates in these headwater-stream ecosystems, and increased mobilization of C resulting from nutrient enrichment is likely to exacerbate the effects of these consumer interactions on downstream ecosystems

(Benstead et al. 2009). Changes in nutrient content of food resources (in our study, primarily on CPOM substrates and greater for P than N) have the potential to drive shifts in detrital foodweb pathways.

#### **Acknowledgements**

We thank Keller Suberkropp and Vladislav Gulis for assistance with ergosterol analyses, and Mary Freeman, Cary Tuckfield, and the University of Georgia Statistics Department for assistance with data analyses. Nutrient analyses were conducted at the Analytical Chemistry Laboratory at the University of Georgia. The manuscript was improved with comments from Vladislav Gulis, Jonathan Benstead, John Davis, 2 anonymous referees, and Associate Editors Eugènia Martí and Michelle Baker. This study and preparation of this manuscript were supported by National Science Foundation grants DEB-0318063 (to ADR, K. Suberkropp, B. Wallace, and M. Black) and DEB-0918894 (to ADR, J. Benstead, V. Gulis, and J. Maerz) and an Odum School of Ecology Graduate Research grant to CJT.

#### **Literature Cited**

- APHA (AMERICAN PUBLIC HEALTH ASSOCIATION). 1998. Standard methods for the examination of water and wastewater. 20<sup>th</sup> edition. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC.
- AUFDENKAMPE, A. K., E. MAYORGA, P. A. RAYMOND, J. M. MELACK, S. C. DONEY, AND S. R. ALIN. 2011. Riverine coupling of biogeochemical cycles between land, oceans, and atmosphere. *Frontiers in Ecology and the Environment* 9:53–60.
- BENSTEAD, J. P., A. D. ROSEMOND, W. F. CROSS, J. B. WALLACE, S. L. EGGERT, K. SUBERKROPP, V. GULIS, J. L. GREENWOOD, AND C. J. TANT. 2009. Nutrient enrichment alters storage and fluxes of detritus in a headwater stream ecosystem. *Ecology* 90:2556–2566.
- BUESING, N., AND M. O. GESSNER. 2002. Comparison of detachment procedures for direct counts of bacteria associated with sediment particles, plant litter and epiphytic biofilms. *Aquatic Microbial Ecology* 27:29–36.
- CHUNG, N., AND K. SUBERKROPP. 2009. Contribution of fungal biomass to the growth of the shredder, *Pycnopsyche gentilis* (Trichoptera: Limnephilidae). *Freshwater Biology* 54:2212–2229.
- COLE, J. J., Y. T. PRAIRIE, N. F. CARACO, W. H. MCDOWELL, L. J. TRANVIK, R. G. STRIEGL, C. M. DUARTE, P. KORTELAINEN, J. A. DOWNING, J. J. MIDDELBURG, AND J. MELACK. 2007. Plumbing the global carbon cycle: integrating inland waters into the terrestrial carbon budget. *Ecosystems* 10: 171–184.
- CROSS, W. F., J. P. BENSTEAD, P. C. FROST, AND S. A. THOMAS. 2005. Ecological stoichiometry in freshwater benthic

- systems: recent progress and perspectives. *Freshwater Biology* 50:1895–1912.
- CROSS, W. F., J. P. BENSTEAD, A. D. ROSEMOND, AND J. B. WALLACE. 2003. Consumer–resource stoichiometry in detritus-based streams. *Ecology Letters* 6:721–732.
- DAVIS, J. M., A. D. ROSEMOND, S. L. EGGERT, W. F. CROSS, AND J. B. WALLACE. 2010. Nutrient enrichment differentially affects body sizes of primary consumers and predators in a detritus-based stream. *Limnology and Oceanography* 55:2305–2316.
- DODDS, W. K. 2007. Trophic state, eutrophication and nutrient criteria in streams. *Trends in Ecology and Evolution* 22:669–676.
- FINDLAY, S., J. TANK, S. DYE, H. M. VALETT, P. J. MULHOLLAND, W. H. MCDOWELL, S. L. JOHNSON, S. K. HAMILTON, J. EDMONDS, W. K. DODDS, AND W. B. BOWDEN. 2002. A cross-system comparison of bacterial and fungal biomass in detritus pools of headwater streams. *Microbial Ecology* 43: 55–66.
- FIRST, M. R., AND J. T. HOLLIBAUGH. 2008. Protistan bacterivory and benthic microbial biomass in an intertidal creek mudflat. *Marine Ecology Progress Series* 361:59–68.
- FROST, P. C., J. P. BENSTEAD, W. F. CROSS, H. HILLEBRAND, J. H. LARSON, M. A. XENOPOULOS, AND T. YOSHIDA. 2006. Threshold elemental ratios of carbon and phosphorus in aquatic consumers. *Ecology Letters* 9:774–779.
- GESSNER, M. O., AND E. CHAUVET. 1993. Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. *Applied and Environmental Microbiology* 59:502–507.
- GREENWOOD, J. L., A. D. ROSEMOND, J. B. WALLACE, W. F. CROSS, AND H. S. WEYERS. 2007. Nutrients stimulate leaf breakdown rates and detritivore biomass: bottom-up effects via heterotrophic pathways. *Oecologia (Berlin)* 151:637–649.
- GULIS, V., A. D. ROSEMOND, K. SUBERKROPP, H. S. WEYERS, AND J. P. BENSTEAD. 2004. Effects of nutrient enrichment on the decomposition of wood and associated microbial activity in streams. *Freshwater Biology* 49:1437–1447.
- GULIS, V., AND K. SUBERKROPP. 2003. Leaf litter decomposition and microbial activity in nutrient-enriched and unaltered reaches of a headwater stream. *Freshwater Biology* 48:123–134.
- HURLBERT, S. H. 1984. Pseudoreplication and the design of ecological field experiments. *Ecological Monographs* 54: 187–211.
- LISLE, J. T., AND J. C. PRISCU. 2004. The occurrence of lysogenic bacteria and microbial aggregates in the lakes of the McMurdo Dry Valleys, Antarctica. *Microbial Ecology* 47:427–439.
- MACK, M. C., E. A. G. SCHUUR, M. S. BRET-HARTE, G. R. SHAVER, AND F. S. CHAPIN. 2004. Ecosystem carbon storage in arctic tundra reduced by long-term nutrient fertilization. *Nature* 431:440–443.
- NEWELL, S. Y., T. L. ARSUFFI, AND R. D. FALLON. 1988. Fundamental procedures for determining ergosterol content of decaying plant-material by liquid-chromatography. *Applied and Environmental Microbiology* 54: 1876–1879.
- NOBLE, R. T., AND J. A. FUHRMAN. 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquatic Microbial Ecology* 14:113–118.
- PASCOAL, C., F. CASSIO, A. MARCOTEGUI, B. SANZ, AND P. GOMES. 2005. Role of fungi, bacteria, and invertebrates in leaf litter breakdown in a polluted river. *Journal of the North American Benthological Society* 24:784–797.
- ROBINSON, C. T., AND M. O. GESSNER. 2000. Nutrient addition accelerates leaf breakdown in an alpine springbrook. *Oecologia (Berlin)* 122:258–263.
- ROSEMOND, A. D., W. F. CROSS, J. L. GREENWOOD, V. GULIS, S. L. EGGERT, K. SUBERKROPP, J. B. WALLACE, AND S. E. DYE. 2008. Nitrogen versus phosphorus demand in a detritus-based headwater stream: what drives microbial to ecosystem response? *Verhandlungen der Internationalen Vereinigung für theoretische und angewandte Limnologie* 30:651–655.
- ROSEMOND, A. D., C. M. SWAN, J. S. KOMINOSKI, AND S. E. DYE. 2010. Non-additive effects of litter mixing are suppressed in a nutrient-enriched stream. *Oikos* 119:326–336.
- ROSI-MARSHALL, E. J., AND J. B. WALLACE. 2002. Invertebrate food webs along a stream resource gradient. *Freshwater Biology* 47:129–141.
- SMITH, V. H., AND D. W. SCHINDLER. 2009. Eutrophication science: where do we go from here? *Trends in Ecology and Evolution* 24:201–207.
- STELZER, R. S., J. HEFFERNAN, AND G. E. LIKENS. 2003. The influence of dissolved nutrients and particulate organic matter quality on microbial respiration and biomass in a forest stream. *Freshwater Biology* 48:1925–1937.
- SUBERKROPP, K., AND H. WEYERS. 1996. Application of fungal and bacterial production methodologies to decomposing leaves in streams. *Applied and Environmental Microbiology* 62:1610–1615.
- SUBERKROPP, K., V. GULIS, A. D. ROSEMOND, AND J. P. BENSTEAD. 2010. Ecosystem and physiological scales of microbial responses to nutrients in a detritus-based stream: Results of a 5-year continuous enrichment. *Limnology and Oceanography* 55:149–160.
- SWANK, W. T., AND D. A. CROSSLEY. 1988. *Forest hydrology and ecology at Coweeta*. Springer-Verlag, New York.
- WALLACE, J. B., S. L. EGGERT, J. L. MEYER, AND J. R. WEBSTER. 1997. Multiple trophic levels of a forest stream linked to terrestrial litter inputs. *Science* 277:102–104.
- WARD, G. M., A. M. WARD, C. N. DAHM, AND N. G. AUMEN. 1994. Origin and formation of organic and inorganic particles in aquatic systems. Pages 45–73 in R. S. Wotton (editor). *The biology of particles in aquatic systems*. Lewis Publishers, Boca Raton, FL.
- WEBSTER, J. R., AND E. F. BENFIELD. 1986. Vascular plant breakdown in freshwater ecosystems. *Annual Review of Ecology and Systematics* 17:567–594.
- WOTTON, R. S. 1994. Particulate and dissolved organic matter as food. Pages 235–288 in R. S. Wotton (editor). *The biology of particles in aquatic systems*. Lewis Publishers, Boca Raton, Florida.

Received: 30 March 2012

Accepted: 24 July 2013