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## Low-to-moderate nitrogen and phosphorus concentrations accelerate microbially driven litter breakdown rates

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# Low-to-moderate nitrogen and phosphorus concentrations accelerate microbially driven litter breakdown rates

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**Abstract.** Particulate organic matter (POM) processing is an important driver of aquatic ecosystem productivity that is sensitive to nutrient enrichment and drives ecosystem carbon (C) loss. Although studies of single concentrations of nitrogen (N) or phosphorus (P) have shown effects at relatively low concentrations, responses of litter breakdown rates along gradients of low-to-moderate N and P concentrations are needed to establish likely interdependent effects of dual N and P enrichment on baseline activity in stream ecosystems. We established 25 combinations of dissolved inorganic N (DIN; 55–545 µg/L) and soluble reactive P (SRP; 4–86 µg/L) concentrations with corresponding N:P molar ratios of 2–127 in experimental stream channels. We excluded macroinvertebrates, focusing on microbially driven breakdown of maple (*Acer rubrum* L.) and rhododendron (*Rhododendron maximum* L.) leaf litter. Breakdown rates,  $k$ , per day ( $d^{-1}$ ) and per degree-day ( $dd^{-1}$ ), increased by up to 6× for maple and 12× for rhododendron over our N and P enrichment gradient compared to rates at low ambient N and P concentrations. The best models of  $k$  ( $d^{-1}$  and  $dd^{-1}$ ) included litter species identity and N and P concentrations; there was evidence for both additive and interactive effects of N and P. Models explaining variation in  $k$   $dd^{-1}$  were supported by N and P for both maple and rhododendron ( $R_{adj}^2 = 0.67$  and  $0.33$ , respectively). Residuals in the relationship between  $k$   $dd^{-1}$  and N concentration were largely explained by P, but residuals for  $k$   $dd^{-1}$  and P concentration were less adequately explained by N. Breakdown rates were more closely related to nutrient concentrations than variables associated with measurements of two mechanistic parameters associated with C loss (fungal biomass and microbial respiration rate). We also determined the effects of nutrient addition on litter C:nutrient stoichiometry and found reductions in litter C:N and C:P along our experimental nutrient gradient. Our results indicate that microbially driven litter processing rates increase across low-to-moderate nutrient gradients that are now common throughout human-modified landscapes.

**Key words:** carbon processing; detritus; ecological stoichiometry; ecosystem function; litter breakdown; nutrient enrichment; organic matter quality; streams.

## INTRODUCTION

Aquatic ecosystem dynamics are driven by important detrital as well as algal resource pathways, both of which are affected by excessive nutrient loading. In streams, nutrient inputs affect consumers via changes in the quantity, quality, and composition of algal resources (Evans-White et al. 2009, Taylor et al. 2014). Detrital resource quality also changes in response to nutrient inputs, but its quantitative responses are fundamentally different from those of algae and other primary producers: detrital standing crops typically decrease in response to nutrient enrichment (Benstead et al. 2009).

Concepts of aquatic ecosystem health consequently must be expanded to include both algal and detrital responses to nutrient enrichment (Dodds and Cole 2007, Palmer and Febria 2012).

Few studies have tested the effects of both N and P on detrital resources at concentrations associated with current watershed land use change. Studies in which nitrogen (N) and phosphorus (P) effects on detrital carbon dynamics (specifically effects on litter mass loss rates) were tested typically used single-concentration combinations of N and P (Gulis and Suberkropp 2003, Greenwood et al. 2007, Benstead et al. 2009) or landscape or experimental gradients in N or P (P, Rosemond et al. [2002]; N, Ferreira et al. [2006]; N and P, Pascoal et al. [2003], Woodward et al. [2012]). Landscape gradients establish more realistic ranges for loss rates, but often incorporate confounding factors, such as flow, that can vary among sites, or pollution

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effects that covary with nutrient concentrations. When gradients in N (experimental; Ferreira et al. [2006]) or P (landscape; Rosemond et al. [2002]) have been used to determine responses of heterotrophic pathways without such confounding effects, large responses in detrital processing rates have been observed over relatively narrow ranges of N or P concentrations. However, dissolved N and P concentrations typically covary across land-use gradients (e.g., Taylor et al. 2014) and the relative importance and interactive effects of N and P on detrital processing rates are less clear.

We used a crossed concentration-gradient design in experimental stream channels to test simultaneous effects of N and P on litter breakdown at multiple, low to moderate concentrations. We focused on microbially driven carbon (C) processing because microbes (aquatic fungi and, to a lesser extent, bacteria) are the “first line” of response of stream detrital pathways to nutrient enrichment, which typically stimulates microbial rates of growth, production, and litter-associated respiration (Suberkropp and Chauvet 1995, Gulis et al. 2008, Suberkropp et al. 2010). We tested whether microbially mediated litter breakdown was N or P limited, whether responses to a single nutrient depended on the availability of the other (i.e., co-limitation), and how response magnitudes were dependent on litter species identity and quality (initial C:N and C:P ratios). We tested the effects of N and P enrichment on microbial biomass (fungal biomass) and activity (respiration) and compared those responses to the more integrative measure of litter breakdown to examine which variables responded most predictably to N and P concentration gradients. We also tested for the effects of N and P addition on litter nutrient content (C:N, C:P), due to the increased importance of invertebrate detritivores in accelerating litter loss rates when C:N and C:P are reduced (Hladyz et al. 2009, Rosemond et al. 2010). The dissolved nutrient concentrations we used reflect a range of landscape conditions that would include relatively pristine to moderately impacted streams in the United States and Europe (Alexander and Smith 2006, Woodward et al. 2012), a range over which experimental additions of both N and P have not been previously tested within a single study. We predicted that added N and P would result in additive increases in microbial processing of litter, such that litter breakdown rates would be highest at the highest concentrations of N and P, and we expected higher relative increases in breakdown rates for litter species with higher initial C:N and C:P. We also predicted additive effects of added N and P on microbial respiration rates and fungal biomass.

## METHODS

### *Study site and experimental channels*

We conducted our experiment at the Coweeta Hydrologic Laboratory (CWT), a United States Forest

Service (USFS) and Long-term Ecological Research (LTER) site located in Macon County, North Carolina, USA (35°03'35" N, 83°25'48" W). CWT is located in the Blue Ridge Province of the southern Appalachian Mountains at approximately 693 m above sea level. Stream water was pumped from a third-order stream (Shope Fork) into a 1500-L tank that supplied three 378-L header tanks. Water entered aluminum stream channels (0.15 × 0.15 × 4 m;  $n = 26$ ) through adjustable spouts extending from the header tanks to the top of each stream channel. Channels lacked substratum to reduce differential nutrient uptake associated with any possible differences in benthic complexity. Nutrient enrichment relative to ambient dissolved inorganic nitrogen (DIN;  $\text{NO}_3^-$ -N +  $\text{NH}_4^+$ -N) and soluble reactive phosphorus (SRP) concentrations in Shope Fork water was achieved by continuously dosing 25 combinations of N ( $\text{NH}_4\text{NO}_3$ ) and P ( $\text{H}_3\text{PO}_4$ ) using high-accuracy, multichannel peristaltic pumps (Watson-Marlow, Wilmington, Massachusetts, USA). Our target concentrations were represented by five levels of elevated DIN across a gradient of 81–650  $\mu\text{g/L}$  and five concentrations of elevated SRP across a gradient of 11–90  $\mu\text{g/L}$ . These concentrations represent <10th to >50th percentiles of DIN and SRP in U.S. rivers (Alexander and Smith 2006). Each target concentration of P was crossed with low to high concentrations of N and vice versa, resulting in 25 unique combinations of N and P concentration and a range in N:P of 2 to 127 (Table 1). One stream channel served as an ambient control, and received stream water from Shope Fork only (mean DIN = 55  $\mu\text{g/L}$ ; mean SRP = 4  $\mu\text{g/L}$ ; mean N:P = 27.5; Table 1).

### *Experimental conditions*

Physical parameters, including water depth (approximately 0.1 m) were standardized among all channels. Discharge within stream channels was adjusted weekly to maintain a consistent 0.1 L/s rate in all channels. Fine sediment was flushed from channels weekly to reduce potential for anoxic conditions and differential microbial uptake of nutrients. All channels were covered with shade cloth to maintain consistent light levels that were similar to forested streams at CWT. Water temperature was recorded every 30 min by submersible temperature probes ( $n = 3$  channels; Onset Computer, Pocasset, Massachusetts, USA).

Water samples were collected weekly from the midpoint of each stream channel, filtered through Millipore nitrocellulose filters (nominal pore size 0.45  $\mu\text{m}$ ), transported to the laboratory on ice and frozen. Samples were subsequently thawed and analyzed for  $\text{NH}_4^+$ -N (automated phenate colorimetry) and  $\text{NO}_3^-$ -N (cadmium reduction followed by automated colorimetry) with an Alpkem 300 Autoanalyzer (College Station, Texas, USA). Phosphorus concentrations were determined spectrophotometrically (ascorbic acid

TABLE 1. Crossed gradient of five concentrations ( $\mu\text{g/L}$ ) of dissolved inorganic nitrogen (DIN;  $\text{NO}_3^- \text{-N} + \text{NH}_4^+ \text{-N}$ ), soluble reactive phosphorus (SRP), and DIN:SRP (N:P) molar ratios in experimental stream channels ( $n = 25$ ) at Coweeta Hydrologic Laboratory, Macon County, North Carolina, USA.

Treatment	Targeted			Measured					
	DIN	SRP	N:P	DIN		SRP		N:P	
				Mean	% $\Delta$	Mean	% $\Delta$	Mean	% $\Delta$
Control	NA	NA	NA	54.5 (11.5)	NA	4.4 (1.7)	NA	27	NA
N <sub>1</sub> P <sub>1</sub>	81.3	11.3	16	54.9 (19.1)	-32	10.0 (2.0)	-12	12	-25
N <sub>1</sub> P <sub>2</sub>	81.3	33.8	5	55.2 (21.2)	-32	27.4 (3.2)	-19	4	-20
N <sub>1</sub> P <sub>3</sub>	81.3	50.6	4	101.7 (41.0)	25	36.2 (4.2)	-28	6	-50
N <sub>1</sub> P <sub>4</sub>	81.3	67.5	3	32.1 (15.5)	-61	44.4 (6.8)	-34	2	-33
N <sub>1</sub> P <sub>5</sub>	81.3	90	2	31.6 (15.2)	-61	77.3 (7.9)	-14	1	-50
N <sub>2</sub> P <sub>1</sub>	243.9	11.3	48	146.0 (22.9)	-40	9.4 (1.5)	-17	34	-29
N <sub>2</sub> P <sub>2</sub>	243.9	33.8	16	177.6 (32.7)	-27	33.7 (4.4)	0	12	-25
N <sub>2</sub> P <sub>3</sub>	243.9	50.6	11	140.9 (42.6)	-42	47.3 (7.1)	-7	7	-36
N <sub>2</sub> P <sub>4</sub>	243.9	67.5	8	258.9 (41.3)	6	67.2 (9.0)	0	9	13
N <sub>2</sub> P <sub>5</sub>	243.9	90	6	153.4 (38.1)	-37	85.6 (7.9)	-5	4	-33
N <sub>3</sub> P <sub>1</sub>	365.8	11.3	72	283.8 (69.9)	-22	12.7 (2.8)	12	49	-32
N <sub>3</sub> P <sub>2</sub>	365.8	33.8	24	331.7 (71.3)	-9	42.0 (7.7)	24	17	-29
N <sub>3</sub> P <sub>3</sub>	365.8	50.6	16	189.5 (39.4)	-48	44.0 (4.3)	-13	10	-38
N <sub>3</sub> P <sub>4</sub>	365.8	67.5	12	246.0 (38.5)	-33	57.4 (7.4)	-15	9	-25
N <sub>3</sub> P <sub>5</sub>	365.8	90	9	218.7 (41.0)	-40	77.7 (8.7)	-14	6	-33
N <sub>4</sub> P <sub>1</sub>	487.7	11.3	96	293.3 (44.7)	-40	7.7 (1.1)	-31	85	-11
N <sub>4</sub> P <sub>2</sub>	487.7	33.8	32	301.8 (39.5)	-38	25.7 (1.8)	-24	26	-19
N <sub>4</sub> P <sub>3</sub>	487.7	50.6	21	445.5 (102.0)	-9	47.4 (5.9)	-6	21	0
N <sub>4</sub> P <sub>4</sub>	487.7	67.5	16	209.7 (36.8)	-57	57.4 (4.4)	-15	8	-50
N <sub>4</sub> P <sub>5</sub>	487.7	90	12	283.3 (54.9)	-42	67.3 (4.2)	-25	9	-25
N <sub>5</sub> P <sub>1</sub>	650.3	11.3	127	400.1 (44.8)	-38	9.2 (1.7)	-19	97	-24
N <sub>5</sub> P <sub>2</sub>	650.3	33.8	43	544.8 (134.0)	-16	46.5 (12.4)	38	26	-40
N <sub>5</sub> P <sub>3</sub>	650.3	50.6	28	508.2 (130.5)	-22	47.4 (13.2)	-6	24	-14
N <sub>5</sub> P <sub>4</sub>	650.3	67.5	21	461.7 (97.1)	-29	69.8 (11.0)	3	15	-29
N <sub>5</sub> P <sub>5</sub>	650.3	90	16	305.3 (84.5)	-53	68.5 (8.8)	-24	10	-38

Notes: Measured concentrations are means (with SE in parentheses) from weekly water samples collected throughout the experiment ( $n = 7$  replicates). The percent difference between targeted and measured nutrient concentrations and molar N:P ratios is shown as % $\Delta$ . NA, not applicable.

method [APHA 1998]; UV-1700, Shimadzu, Tokyo, Japan).

#### Litter breakdown rates

Breakdown rates of red maple (*Acer rubrum* L.) and rhododendron (*Rhododendron maximum* L.) leaf litter were determined during late summer to early autumn 2011. These litter species have different chemical composition (C:N, C:P) and physical attributes (e.g., a waxy cuticle on rhododendron), resulting in different breakdown rates under a variety of field conditions (Kominoski et al. 2007, Ardón et al. 2009). Fine-mesh bags (500- $\mu\text{m}$  mesh) were used to exclude macroinvertebrates. Litter bags ( $n = 208$ ) containing air-dried maple or rhododendron leaf litter ( $1.1 \pm 0.07$  g [mean  $\pm$  SE]), were deployed in the stream channels on 23 August 2011. Ten litter bags (five of each species) were used to measure handling losses and initial litter chemistry. Litter bags ( $n = 52$ , two per channel on each date) were collected after 7, 14, 28, and 59 d of incubation, returned to the lab on ice, and processed within 12 h. Retrieved leaf litter was rinsed to remove debris, oven-dried at 60°C, and a subsample combusted at 550°C to calculate ash-free dry mass (AFDM). On days 14 and 59, subsamples of leaf litter were analyzed for microbial respiration, fungal biomass, and elemental stoichiometry

(see *Litter nutrient content and elemental stoichiometry*).

Leaf breakdown rates were expressed as the decay rate coefficient,  $k$ , per day ( $k \text{ d}^{-1}$ ) and per degree-day ( $k \text{ dd}^{-1}$ ) in the exponential model  $m_t/m_0 = e^{-kt}$ , where  $m_0$  is the initial AFDM and  $m_t$  is AFDM at time  $t$ . The exponential model  $m_t/m_0 = e^{-k\text{dd}}$  was used to calculate  $k \text{ dd}^{-1}$ , where dd is degree-days. We tested the appropriateness of this model for nutrient and leaf litter species effects using goodness of fit ( $R^2 > 0.50$ ).

#### Microbial respiration rates and fungal biomass

Microbial respiration rates were measured as oxygen uptake of decomposing litter at stream water temperatures (Gulis and Suberkropp 2003). Multiple fragments of leaf litter were collected from each litter bag within 12 h of retrieval and placed in filtered stream water in respiration chambers (30 mL), in an incubator set at stream temperature. Oxygen concentrations were recorded every 5–7 min with YSI 5100 Dissolved Oxygen meters (Yellow Springs, Ohio, USA) for 30 min in darkness. Additional chambers ( $n = 5$ ) containing only Shope Fork stream water served as controls. Oxygen consumption was determined from the slope of the regression of oxygen concentration against time minus the control slope and expressed per gram leaf AFDM per hour. Leaf

litter was placed in labeled, pre-weighed vials and frozen ( $-20^{\circ}\text{C}$ ) until analyzed for fungal biomass.

Frozen leaf litter from microbial respiration trials was freeze-dried and weighed to obtain dry mass. Fungal biomass associated with these leaf litter samples was estimated from ergosterol concentrations. Lipids were extracted in methanolic KOH, partitioned into pentane, and re-dissolved in methanol (Newell et al. 1988; as modified by Gulis et al. 2006b). Ergosterol was further purified and quantified by HPLC (Shimadzu) equipped with a Phenomenex Kinetex C18 column (Torrance, California, USA) and a UV detector set at 282 nm. External ergosterol standards (Acros Organics, Geel, Belgium) were used. To convert ergosterol concentration to fungal biomass, we assumed an ergosterol concentration of  $5.5 \mu\text{g}/\text{mg}$  of mycelial dry mass (Gessner and Chauvet 1993).

#### *Litter nutrient content and elemental stoichiometry*

Leaf litter elemental stoichiometry (C:N and C:P) was measured from maple and rhododendron litter on days 0 (initial), 14, and 59. Oven-dried ( $60^{\circ}\text{C}$  for 48 h) litter was ground using a ball mill (Spex Certiprep 8000-D, Metuchen, New Jersey, USA) prior to analyses. C and N content of litter was estimated as a percentage of AFDM, using a Carlo Erba 1500N CHN Analyzer (Carlo Erba, Milan, Italy). Litter for P analysis was weighed in acid-washed, pre-ashed ceramic crucibles, combusted at  $500^{\circ}\text{C}$ , digested in acid, and analyzed spectrophotometrically (ascorbic acid method; APHA 1998). Phosphorus concentrations in litter were expressed as a percentage of AFDM. All litter stoichiometry data are presented as molar ratios.

#### *Data analysis*

Linear fixed effects models were used to assess effects of leaf litter species (maple, rhododendron), dissolved nutrient concentrations (N and P), and  $\text{N} \times \text{P}$  interaction on response variables (litter  $k$ , fungal biomass, microbial respiration rate, and litter stoichiometry). Interaction of  $\text{N} \times \text{P}$  was included in models with N and P to determine the interdependence of the effects of nutrient concentrations on microbial litter processing. Adding parameters to models can increase likelihood of selection, resulting in overfitting of models. Akaike's information criterion adjusted for small sample size ( $\text{AIC}_c$ ) was used for model selection to determine the least amount of information needed to explain variation in response variables (see above). Models with  $\Delta \text{AIC}_c \leq 4$  were considered equivalent (Burnham and Anderson 2002).

Litter breakdown rates, microbial respiration, fungal biomass, and litter stoichiometry data were compared against log-transformed dissolved N and P concentrations and non-transformed N:P molar ratios using single or multiple linear regressions. The dual control of N and P in driving breakdown rates was also tested by regressing concentrations of N or P against breakdown rates and then plotting residuals of those regressions

against concentration of the other nutrient. We also plotted  $k \text{ dd}^{-1}$  against dual gradients of N and P (i.e., in three-dimensional plots). Saturation response curves (following a Michaelis-Menten form) were fit to maple and rhododendron litter breakdown rates ( $k \text{ d}^{-1}$ ) to estimate the half-saturation constant of DIN and SRP concentrations ( $K_m$ ) whereby the process rate is half of  $V_{\text{max}}$  (the maximum breakdown rate). All statistical analyses were performed using R version 2.14.2 (R Development Core Team 2012).

## RESULTS

### *Experimental conditions*

Measured concentrations of DIN (range =  $32\text{--}541 \mu\text{g}/\text{L}$ ) and SRP (range =  $13\text{--}86 \mu\text{g}/\text{L}$ ) in treatment channels closely matched target concentrations (DIN range =  $83\text{--}650 \mu\text{g}/\text{L}$ ; SRP range =  $11\text{--}90 \mu\text{g}/\text{L}$ ) throughout the experiment (Table 1). In general, greater percent differences in targeted vs. measured concentrations were detected in DIN than in SRP (Table 1). DIN and SRP concentrations in the control channel were variable throughout the experiment but remained consistently lower than those in all treatment channels (Table 1).

Mean daily temperature in stream channels ranged from  $19.1^{\circ}\text{C}$  to  $25.4^{\circ}\text{C}$  with a mean  $\pm$  SE of  $23.0^{\circ} \pm 0.2^{\circ}\text{C}$  throughout the study period. During that same period, mean daily temperature in Shope Fork (source water for stream channels) ranged from  $10.2^{\circ}\text{C}$  to  $18.6^{\circ}\text{C}$  with a mean of  $15.1^{\circ}\text{C}$  ( $\pm 0.3^{\circ}\text{C}$ ).

### *Litter breakdown rates*

Best models of breakdown rates ( $k \text{ d}^{-1}$  and  $k \text{ dd}^{-1}$ ) of maple and rhododendron litter included litter species identity, dissolved N and P concentration, and  $\text{N} \times \text{P}$  interaction, which provides evidence for both additive and interactive effects of N and P (Table 2). Breakdown rates ( $k \text{ dd}^{-1}$ ) increased up to 6 $\times$  for maple and 12 $\times$  for rhododendron over the  $\text{N} \times \text{P}$  gradient (Fig. 1). Breakdown rates increased along both N and P concentration gradients, and higher N and P concentrations interacted, leading to the greatest increases in  $k \text{ dd}^{-1}$  (Fig. 2). Additive effects of N and P were further supported by residual plots of N vs.  $k \text{ dd}^{-1}$  against P; however, residuals of P vs.  $k \text{ dd}^{-1}$  were less affected by N (Appendix A).

Litter  $k$  was moderately affected by dissolved N:P ratios, whereby  $k$  trended toward higher values at  $\text{N:P} < 16$  (maple  $k \text{ d}^{-1} = 0.018 \pm 0.001$ ,  $k \text{ dd}^{-1} = 0.0008 \pm 0.0001$ ; rhododendron  $k \text{ d}^{-1} = 0.008 \pm 0.001$ ,  $k \text{ dd}^{-1} = 0.0003 \pm 0.0001$  [values are means  $\pm$  SE]) than  $\text{N:P} > 16$  (maple  $k \text{ d}^{-1} = 0.016 \pm 0.002$ ,  $k \text{ dd}^{-1} = 0.0007 \pm 0.0002$ ; rhododendron  $k \text{ d}^{-1} = 0.006 \pm 0.002$ ,  $k \text{ dd}^{-1} = 0.0003 \pm 0.0002$ ). However, there was stronger evidence for co-limitation than effects of nutrient ratio per se; there were subtle but consistent trends for the highest breakdown rates for any given P concentration to occur at the highest N:P ratios and for the highest breakdown rates for any given N concentration to occur at the lowest N:P

TABLE 2. Linear fixed-effects models and model weights comparing nitrogen (N) and phosphorus (P) concentrations ( $\mu\text{g/L}$ ,  $\log_{10}$ -transformed),  $N \times P$  interaction, and leaf litter species (*Acer rubrum*, *Rhododendron maximum*) effects on leaf litter breakdown rate per day ( $k \text{ d}^{-1}$ ) and per degree-day ( $k \text{ dd}^{-1}$ ), fungal biomass (F), microbial respiration rates (R), and litter stoichiometry (C:N and C:P) on days 14 and 59.

Model	$K$	$\Delta\text{AIC}_c$	$\text{AIC}_c \text{ wt}$	Cum wt	Log likelihood
Leaf litter breakdown rates					
$k \text{ d}^{-1}$					
N + P + Species	5	0.0	0.59	0.59	486.9
N + P + N $\times$ P + Species	6	0.7	0.41	1.00	487.7
$k \text{ dd}^{-1}$					
N + P + N $\times$ P + Species	6	0.0	0.62	0.62	787.1
N + P + Species	5	1.0	0.38	1.00	785.5
Fungal biomass					
Day	3	0.0	0.45	0.45	-480.9
Species + Day	4	1.4	0.22	0.66	-480.5
P + Species + Day	5	2.7	0.12	0.78	-480.0
N + Species + Day	5	2.9	0.11	0.89	-480.1
N + P + Species + Day	6	3.8	0.07	0.95	-479.5
Microbial respiration rates					
N + P + Species + Day	6	0.0	0.36	0.36	36.4
P + Species + Day	5	0.3	0.31	0.67	35.1
N + Species + Day	5	2.2	0.12	0.79	34.2
N + P + N $\times$ P + Species + Day	7	2.3	0.11	0.91	36.4
Species + Day	4	3.8	0.05	0.96	32.3
Litter C:N					
N + P + Species + Day	6	0.0	0.51	0.51	-435.7
P + Species + Day	5	1.2	0.27	0.78	-437.5
N + P + N $\times$ P + Species + Day	7	2.2	0.17	0.95	-435.7
Litter C:P					
N + P + N $\times$ P + Species + Day	7	1.3	0.62	0.62	-895.8
P + Species + Day	5	1.9	0.25	0.87	-899.0
N + P + Species + Day	6	3.1	0.13	1.00	-898.5

Notes: Data are from decomposing leaf litter exposed to five levels of dissolved inorganic nitrogen ( $\text{NO}_3^-$ -N +  $\text{NH}_4^+$ -N; N) and soluble reactive phosphorus (P) concentrations added to stream channels at Coweeta Hydrologic Laboratory, Macon County, North Carolina, USA. Akaike's information criterion adjusted for small sample sizes ( $\text{AIC}_c$ ) was used to identify model parsimony. Number of parameters in each model is  $K$ . The difference in  $\text{AIC}_c$  scores from the top model (lowest  $\text{AIC}_c$ ) is  $\Delta\text{AIC}_c$ .  $\text{AIC}_c \text{ wt}$  is the weighted  $\text{AIC}_c$  score, which is calculated as  $\Sigma\text{AIC}_c / \text{AIC}_c$ .  $\text{AIC} = 2K - 2\ln(L) + K$ ;  $\text{AIC}_c = \text{AIC} + 2K(K+1)/(n-K-1)$ , whereby  $K$  is the number of parameters in the model,  $L$  is the likelihood function for the model, and  $n$  is sample size. Cum wt is the cumulative model weights of evidence. Models with  $\Delta\text{AIC}_c \leq 4$  are considered equivalent (Burnham and Anderson 2002).

ratios (indicating further limitation by N and P, respectively; Table 3).

#### Fungal biomass and microbial respiration

Fungal biomass was consistently higher on maple than on rhododendron litter. Biomass ranged from 6 to 110 mg/g AFDM leaf litter on day 14 and from 10 to 140 mg/g AFDM leaf litter by day 59 (Appendix B). Litter-associated fungal biomass was explained by models that included N and P concentrations, litter species, and time (Table 2).

Microbial respiration rates were similar for both litter species. Rates ranged from 0.03 to nearly 0.60  $\text{mg O}_2 \cdot (\text{g AFDM})^{-1} \cdot \text{h}^{-1}$  on day 14 and from 0.03 to 0.54  $\text{mg O}_2 \cdot (\text{g AFDM})^{-1} \cdot \text{h}^{-1}$  on day 59 (Appendix B). Top models for microbial respiration rate included litter species identity, time, N and P, and  $N \times P$  interaction (Table 2).

#### Litter nutrient content and elemental stoichiometry

Litter stoichiometry (C:N and C:P ratios) changed during breakdown in response to dissolved N and P concentrations and N:P ratios (Table 3; Fig. 3A–D). In general, larger differences were observed between control and treatment C:P than C:N for both litter species (Table 3). Rhododendron C:N decreased with added N from day 14 to day 59 but that response was consistent across all N concentrations. Litter C:P of both species decreased with increasing P concentration by day 14, and rhododendron C:P decreased with increasing P by day 59 (Fig. 3C and D). Models explaining variation in litter stoichiometry included N and P,  $N \times P$  interaction, litter species, and time (Table 2).

#### DISCUSSION

We observed relatively large increases in microbially driven litter processing rates across N and P concentra-

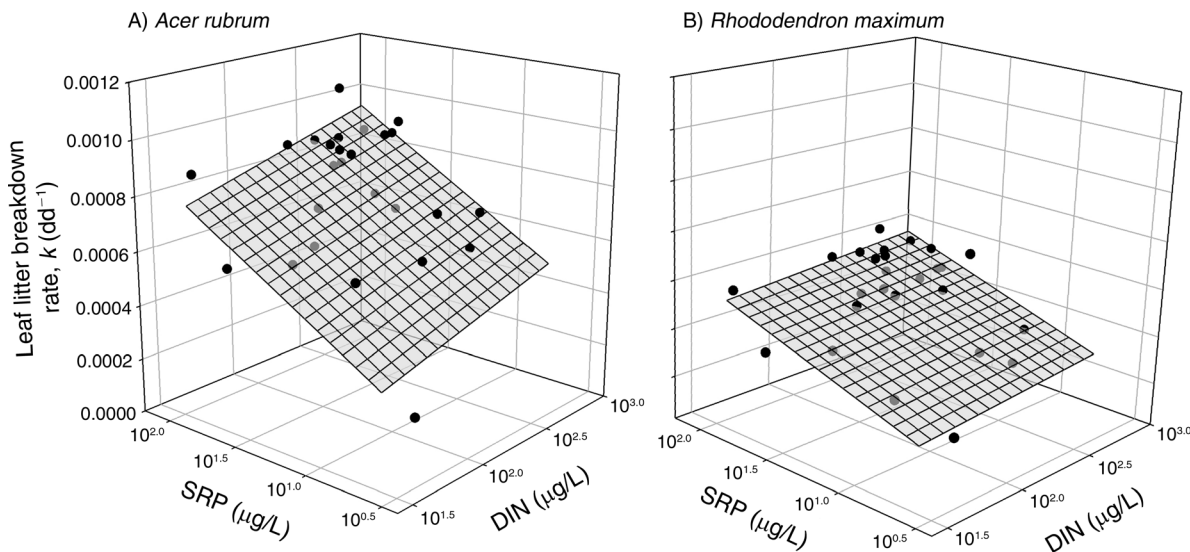


FIG. 1. Surface contour plots of (A) maple (*Acer rubrum*) and (B) rhododendron (*Rhododendron maximum*) leaf litter breakdown rates,  $k$ , per degree-day ( $\text{dd}^{-1}$ ) at different concentrations of dissolved inorganic nitrogen (DIN;  $\text{NO}_3^- \text{-N} + \text{NH}_4^+ \text{-N}$ ) and soluble reactive phosphorus (SRP). Values for DIN and SRP are  $\log_{10}$ -transformed means from weekly water samples collected throughout the study ( $n = 7$  replicates). Adjusted  $R^2$  values for these fits were 0.67 (maple) and 0.33 (rhododendron).

tion gradients that are common throughout virtually pristine to more human-modified landscapes (Alexander and Smith 2006). Even the lowest treatment of N and P concentrations (SRP 11  $\mu\text{g/L}$ , DIN 81  $\mu\text{g/L}$ ) yielded a greater than 3 $\times$  increase in measured breakdown rates over those measured in the control channel (SRP 2  $\mu\text{g/L}$ , DIN 54  $\mu\text{g/L}$ ). At higher concentrations, these rates were quantitatively associated with both N and P concentrations and were also indicative of co-limitation by both nutrients. Our results illustrate potentially strong control by relatively low concentrations of dissolved N and P on breakdown rates of organic matter in stream ecosystems. The changes in litter stoichiometry that we observed due to increased dissolved nutrient availability suggest that under typical field conditions, increased macroinvertebrate feeding would have additional positive effects on litter breakdown rates. A recent pan-European study also showed that microbial responses to N and P occur at relatively low concentrations, whereas macroinvertebrate responses were inhibited at higher concentrations of N and P due to confounding effects of accompanying pollutants found in highly impacted streams (Woodward et al. 2012). Our test in experimental stream channels provides further evidence that litter breakdown is constrained by microbial nutrient limitation (both N and P) at low-to-moderate concentrations. Moreover, the microbially driven changes in litter stoichiometry that we observed suggest that, where macroinvertebrate biomass is not reduced by other pollutants, the indirect stimulation of invertebrate-driven litter decomposition by dissolved nutrients can be an important additional outcome.

Our findings are consistent with other studies that indicate saturating effects of N or P enrichment on

stream C losses at relatively low concentrations (Rosemond et al. 2002, Ferreira et al. 2006, Gulis et al. 2006a, b, Woodward et al. 2012). To test for saturation of processing rates, we applied Michaelis-Menten-type asymptotic models to  $k \text{ d}^{-1}$  (Appendix C), which had similar or better fits than those of our linear models (with the exception of rhododendron  $k \text{ d}^{-1}$  vs. SRP, which had a better linear model fit). Therefore, our results generally suggest that under the conditions tested here, the highest nutrient concentrations we used were close to or above saturation. Our  $K_m$  values for DIN and SRP were roughly consistent with (SRP), or generally lower than (DIN), those measured in other studies. In all studies,  $K_m$  values are generally higher for more recalcitrant litter species. Here,  $K_m$  for DIN was 25 and 31  $\mu\text{g/L}$  for maple and rhododendron, respectively, and these relative low  $K_m$  values could in part be a result of relationships derived from measured DIN concentrations that were lower than targeted additions in stream channels due to uptake (Table 1). Higher N half-saturation constants for litter breakdown rates have been observed in European streams (Ferreira et al. [2006], N half-saturation constants,  $K_m\text{-N}$ , of 183  $\mu\text{g/L}$   $\text{NO}_3\text{-N}$  for alder and 260  $\mu\text{g/L}$   $\text{NO}_3\text{-N}$  for oak litter; Gulis et al. [2006b],  $K_m\text{-N}$  162  $\mu\text{g/L}$   $\text{NO}_3\text{-N}$  for alder litter).  $K_m$  values for SRP were roughly similar in our study (5–15  $\mu\text{g/L}$ ) to values from other systems (lowland streams in Costa Rica [Rosemond et al. 2002],  $K_m\text{-P}$ , 6.5  $\mu\text{g/L}$   $\text{PO}_4^{3-}\text{-P}$ ; mountain streams in Portugal [Gulis et al. 2006a];  $K_m\text{-P}$ , 9–21  $\mu\text{g/L}$  SRP), and could partially explain why measured vs. targeted SRP concentrations were not as low as those for DIN (Table 1). In studies where different litter types were tested,  $K_m$  values also depended on litter type (Gulis et al. 2006a, b).



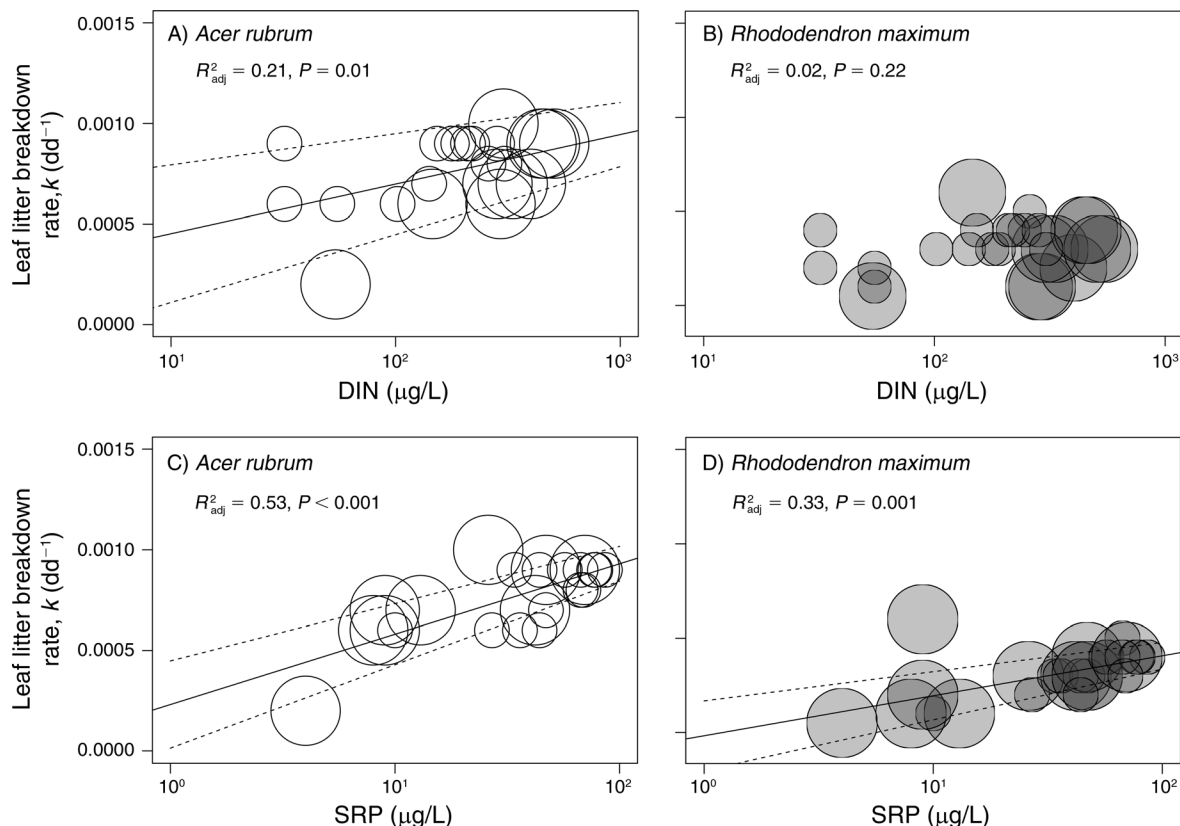


FIG. 2. (A, C) Maple (*Acer rubrum*) and (B, D) rhododendron (*Rhododendron maximum*) leaf litter breakdown rates,  $k$ , per degree-day ( $\text{dd}^{-1}$ ) as a function of dissolved inorganic nitrogen ( $\text{NO}_3^- \text{-N} + \text{NH}_4^+ \text{-N}$ ; DIN) and soluble reactive phosphorus (SRP) concentrations ( $\mu\text{g/L}$ ). Values for DIN and SRP are  $\log_{10}$ -transformed means from weekly water samples collected throughout the study ( $n = 7$  replicates). Bubble size in all plots corresponds to target dissolved N:P molar ratios (small,  $\text{N:P} \leq 16$ ; large,  $\text{N:P} > 16$ ). Solid lines are linear regressions. Dashed lines are 95% confidence intervals. Note that, in three of the four plots, control values (represented by low concentrations of N and P) were lower than predictions based on 95% confidence intervals, indicating that strong responses in breakdown rate were observed at even the lowest additions of N or P. Adjusted (adj)  $R^2$  values and  $P$  values are shown.

Consistent with studies finding that nutrient effects on POM were dependent on litter type, we also found differential responses in the two litter types we tested. Higher-magnitude microbial responses to nutrient enrichment are commonly found for lower-quality litter species, such as oak and rhododendron (Stelzer et al. 2003, Ferreira et al. 2006, Gulis et al. 2006a, Greenwood et al. 2007, Woodward et al. 2012), and we similarly observed stronger effects of N and P on rhododendron  $k$  than on maple  $k$  (Table 3). In addition, the greatest changes in fungal biomass and activity due to nutrient enrichment have been found on wood, which is even lower quality than leaf litter (Gulis et al. 2004, 2008).

Observed reduction of litter C:N and C:P ratios due to elevated dissolved N and P suggest in situ breakdown rates would be accelerated further in the presence of invertebrate detritivores. For example, macroinvertebrate shredder biomass in CWT streams increases with reduced litter C:N (Rosemond et al. 2010), and lower C:N has also been associated with greater invertebrate-

mediated breakdown in other studies (Hladysz et al. 2009). The changes in litter C:N that we observed in this study ( $>150$  to  $<100$ ; Table 3) are consistent with potential thresholds of lower vs. higher macroinvertebrate contributions to breakdown (Rosemond et al. 2010). Likewise, lower litter C:P values observed in this study (Table 3) are consistent with predictions of reduced P-limitation of stream shredders (Tant et al. 2013). We observed higher reductions in litter C:P than C:N (Table 3), suggesting overall greater reductions in P than N limitation for consumers.

Our observations of apparent N and P co-limitation of microbial organic matter processing could be explained by differential responses of fungi and bacteria to N or P. Fungi and bacteria are both important drivers of stream organic matter breakdown, and recent evidence suggests that fungi may be more N limited and bacteria more P limited (V. Gulis, unpublished data) over the range in concentrations that we tested. In a previous whole-stream nutrient enrichment study at Coweeta, fungi comprised 99% and bacteria 95% of total

TABLE 3. Breakdown rates per day ( $k \text{ d}^{-1}$ ) and degree-day ( $k \text{ dd}^{-1}$ ) and litter stoichiometry (C:N and C:P) for maple (*Acer rubrum*) and rhododendron (*Rhododendron maximum*) leaf litter at different targeted concentrations of dissolved inorganic nitrogen (DIN;  $\text{NO}_3^- \text{-N} + \text{NH}_4^+ \text{-N}$ ) and soluble reactive phosphorus (SRP), and DIN:SRP (N:P) ratios on day 59.

Treatment	<i>Acer rubrum</i>						<i>Rhododendron maximum</i>					
	$k \text{ d}^{-1}$	$k \text{ dd}^{-1}$	C:N		C:P		$k \text{ d}^{-1}$	$k \text{ dd}^{-1}$	C:N		C:P	
			Ratio	% $\Delta$	Ratio	% $\Delta$			Ratio	% $\Delta$	Ratio	% $\Delta$
Control	0.004	0.0002	56.4	-	5548.7	-	0.001	0.00005	100.2	-	10276.8	-
N <sub>1</sub> P <sub>1</sub>	0.014	0.0006	49.2	-13	2446.8	-56	0.003	0.0001	98.7	-1	4595.2	-55
N <sub>1</sub> P <sub>2</sub>	0.015	0.0006	33.1	-41	1221.0	-78	0.005	0.0002	69.2	-31	3341.0	-67
N <sub>1</sub> P <sub>3</sub>	0.014	0.0006	45.1	-20	1909.1	-66	0.006	0.0003	55.2	-45	2709.5	-74
N <sub>1</sub> P <sub>4</sub>	0.014	0.0006	34.0	-40	1307.3	-76	0.004	0.0002	115.4	15	3643.5	-65
N <sub>1</sub> P <sub>5</sub>	0.016	0.0009	37.5	-34	1595.5	-71	0.007	0.0004	103.5	3	3391.8	-67
N <sub>2</sub> P <sub>1</sub>	0.015	0.0006	42.8	-24	2713.8	-51	0.009	0.0006	63.0	-37	4877.9	-53
N <sub>2</sub> P <sub>2</sub>	0.020	0.0009	25.2	-55	894.6	-84	0.007	0.0003	76.1	-24	3741.0	-64
N <sub>2</sub> P <sub>3</sub>	0.017	0.0007	29.4	-48	1147.2	-79	0.008	0.0003	94.4	-6	3027.7	-71
N <sub>2</sub> P <sub>4</sub>	0.019	0.0008	37.5	-34	5832.5	5	0.010	0.0005	80.1	-20	2702.4	-74
N <sub>2</sub> P <sub>5</sub>	0.021	0.0009	25.8	-54	830.0	-85	0.010	0.0004	66.4	-34	2474.7	-76
N <sub>3</sub> P <sub>1</sub>	0.015	0.0007	36.1	-36	1775.6	-68	0.003	0.0001	58.7	-41	4146.6	-60
N <sub>3</sub> P <sub>2</sub>	0.016	0.0007	28.3	-50	1251.8	-77	0.006	0.0003	51.3	-49	1860.0	-82
N <sub>3</sub> P <sub>3</sub>	0.022	0.0009	32.9	-42	1556.7	-72	0.006	0.0003	57.8	-42	2550.3	-75
N <sub>3</sub> P <sub>4</sub>	0.024	0.0011	31.2	-45	1087.7	-80	0.009	0.0004	54.6	-46	1703.0	-83
N <sub>3</sub> P <sub>5</sub>	0.020	0.0009	27.5	-51	1312.9	-76	0.009	0.0004	58.6	-42	2103.8	-80
N <sub>4</sub> P <sub>1</sub>	0.014	0.0006	32.3	-43	1490.3	-73	0.003	0.0001	75.4	-25	5144.5	-50
N <sub>4</sub> P <sub>2</sub>	0.022	0.0010	30.7	-46	1255.7	-77	0.006	0.0003	54.0	-46	3629.7	-65
N <sub>4</sub> P <sub>3</sub>	0.022	0.0009	33.7	-40	1928.0	-65	0.010	0.0004	51.6	-49	2061.7	-80
N <sub>4</sub> P <sub>4</sub>	0.020	0.0009	32.6	-42	1415.1	-74	0.009	0.0004	52.9	-47	2073.9	-80
N <sub>4</sub> P <sub>5</sub>	0.020	0.0009	33.5	-41	1411.1	-75	0.010	0.0004	48.0	-52	1946.4	-81
N <sub>5</sub> P <sub>1</sub>	0.017	0.0007	38.6	-32	1615.9	-71	0.005	0.0002	65.3	-35	4887.0	-52
N <sub>5</sub> P <sub>2</sub>	0.013	0.0006	26.3	-53	1189.4	-79	0.007	0.0003	64.0	-36	3392.3	-67
N <sub>5</sub> P <sub>3</sub>	0.020	0.0006	32.8	-42	1623.3	-71	0.007	0.0003	56.9	-43	3041.9	-70
N <sub>5</sub> P <sub>4</sub>	0.020	0.0009	31.4	-44	1106.1	-80	0.009	0.0004	55.7	-44	2083.4	-80
N <sub>5</sub> P <sub>5</sub>	0.019	0.0008	36.2	-36	1631.5	-71	0.007	0.0003	54.1	-46	1775.4	-83

Notes: Data are not replicated ( $n = 1$ ). The percent difference between control and treatment litter C:N and C:P molar ratios is shown as % $\Delta$ .

microbial biomass associated with benthic coarse particulate organic matter (CPOM) and fine particulate organic matter (FPOM), respectively (Tant et al. 2013). Although fungi and bacteria associated with CPOM and FPOM both respond positively to nutrient enrichment (Baldy et al. 2007, Tant et al. 2013), bacteria may continue to increase growth and production rates with nutrient enrichment at elevated temperatures. In this study, water temperatures in our experimental channels were 5–8°C higher than in Shope Fork and as high as 25°C. Although temperature and nutrients can both stimulate fungal activity (Fernandes et al. 2009, Ferreira and Chauvet 2011), increased stream temperatures can decrease fungal sporulation rates and alter fungal species composition (Bärlocher et al. 2008, Fernandes et al. 2009). Thus, the strong effect of P in this study might be partly attributed to higher bacterial processing rates during warmer periods of the experiment. Our higher breakdown rates and greater relative response to nutrient enrichment in experimental stream channels compared to whole-stream experimental nutrient enrichment with macroinvertebrates present (Greenwood et al. 2007, Rosemond et al. 2010) are consistent with temperature, and potentially bacteria, having additional effects on breakdown rates in this study. These comparisons also indicate that the magnitude change in breakdown rates in this study overestimate what has

been observed under natural stream conditions; thus, conservative extrapolation of these results would include the direction, but not the size, of nutrient effects.

Understanding the mechanisms by which N and P enrichment influence ecosystem function is important for establishment of ecologically relevant nutrient criteria in diverse aquatic ecosystems that vary fundamentally in trophic state, biogeochemistry, and human influence (Alexander and Smith 2006). Low-to-moderate nutrient concentrations accelerated microbial processing rates of C and decreased litter C:nutrient ratios, indicating that heterotrophic stream microbes simultaneously respond to both N and P. Therefore, quantifying how ecosystem function changes along gradients in N and P concentrations and N:P ratios is imperative to establish broadly appropriate nutrient criteria among different aquatic ecosystems. Understanding the responses of microbial communities to low-to-moderate nutrient enrichment is important given both the range in N and P concentrations from point and non-point sources found throughout landscapes and the conservation imperative to predict nutrient effects on organic matter quantity and quality throughout river networks (Kominoski and Rosemond 2012). It is still unclear how recent and projected increases in N relative to P throughout U.S. streams and rivers (Alexander and Smith 2006) may affect detrital carbon processing, but

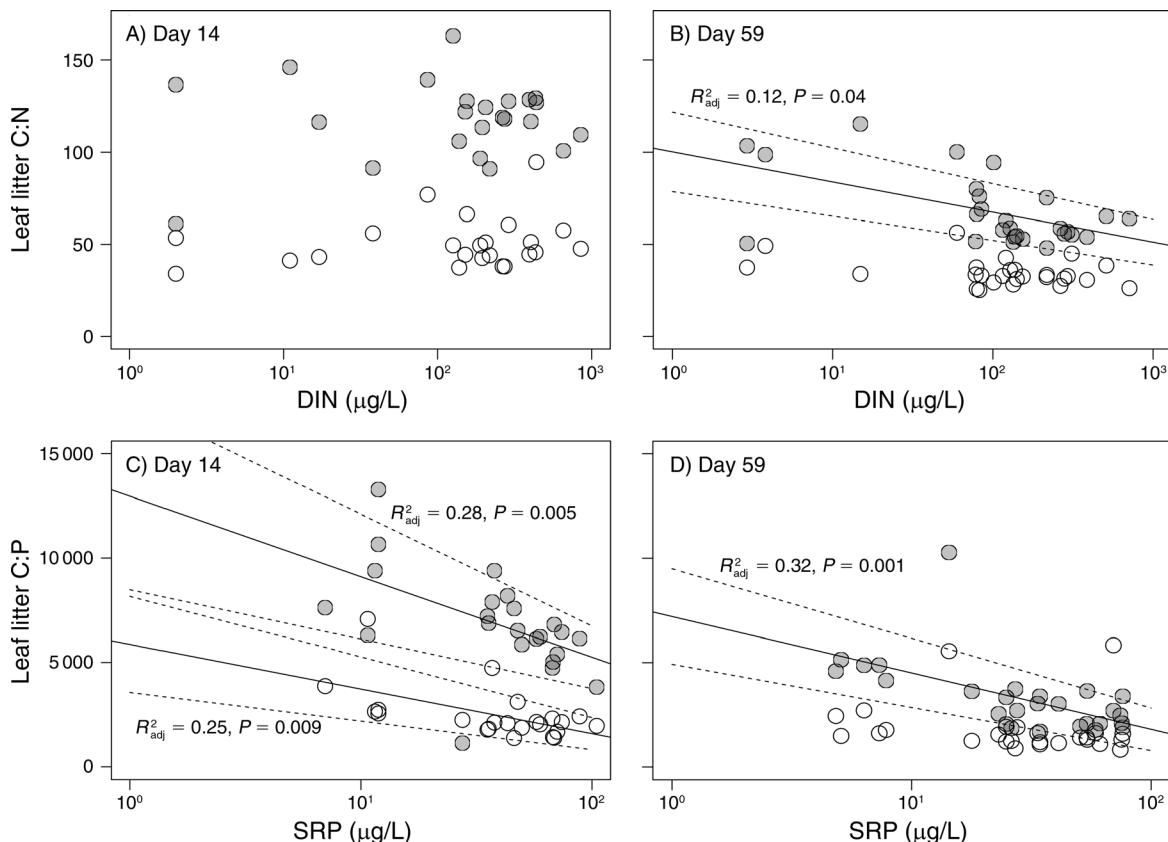


FIG. 3. C:N and C:P ratios of decomposing maple (*Acer rubrum*) and rhododendron (*Rhododendron maximum*) litter incubated for 14 and 59 days along a gradient in (A, B) dissolved inorganic nitrogen ( $\text{NO}_3^-$ -N +  $\text{NH}_4^+$ -N; N) and (C, D) soluble reactive phosphorus (P) concentrations ( $\mu\text{g/L}$ ). Solid lines are linear regressions. Dashed lines are 95% confidence intervals. White circles are for *Acer rubrum*. Gray circles are for *Rhododendron maximum*. Adjusted (adj)  $R^2$  values and  $P$  values are shown.

increases in water temperatures are likely to interact with added nutrients to increase net ecosystem carbon losses (Fernandes et al. 2009, Ferreira and Chauvet 2011).

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#### SUPPLEMENTAL MATERIAL

##### Ecological Archives

Appendices A–C are available online: <http://dx.doi.org/10.1890/14-1113.1.sm>