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Litter Conditioning is Differentially Affected by Leaf Species, Phosphorus Enrichment, and Light Availability

Erin E. Scott
University of Arkansas, Fayetteville

LITTER CONDITIONING IS DIFFERENTIALLY AFFECTED BY LEAF SPECIES, PHOSPHORUS ENRICHMENT, AND LIGHT AVAILABILITY

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Erin E. Scott
University of Arkansas
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ABSTRACT

Anthropogenic enrichment of nitrogen and phosphorus is one of the most pervasive and detrimental threats to aquatic ecosystems worldwide. In streams that rely on allochthonous basal food resources, such as leaves, nutrient pollution can result in altered food quality and decreased carbon (C) standing stocks. However, the magnitude and mechanisms of this change in quality are poorly understood. Laboratory microcosm studies were conducted to 1) quantify the response of litter C:P to a gradient of phosphorus (P) enrichment (0, 0.05, and 0.5 mg SRP/L) across leaf species with variable levels of degradability (sugar maple and oak), and 2) quantify the response of litter C:P to a range of P concentrations (0, 0.05, and 0.5 mg SRP/L) and light availability (15 and 500 µmol photons m^{-2} s^{-1}). Results of the first experiment showed that litter %P increased and C:P decreased with increasing water column P concentrations and this response was greater for the more labile maple species. Carbon:P remained relatively constant through time in the low-P treatments (2600 for both maple and oak) and declined significantly in the high-P treatments (480 and 1040 for maple and oak, respectively). Results of the second experiment demonstrated that phosphorus concentrations and light availability differentially affected algal biomass (as chlorophyll *a*), microbial metabolic rates, and litter stoichiometry. Algal biomass responded to increased P enrichment only when coupled with greater light intensity, and respiration rates increased with P enrichment in both light levels. Litter C:P ratios decreased significantly with P enrichment with a differential response across light intensities. Our results demonstrate the complexities of nutrient pollution on forested stream ecosystem functioning where allochthonous food resources are important. The effects of nutrient enrichment on detrital quality can provide an important link to understanding how nutrient loading impacts aquatic consumers and potential biodiversity losses.

This thesis is approved for recommendation
to the Graduate Council:

Thesis Director:

_____

Dr. J. Thad Scott

Thesis Committee:

_____

Dr. Michelle Evans-White

_____

Dr. Mary Savin

_____

Dr. Brian E. Haggard

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Scott, E. E., C. Prater, E. Norman, B. C. Baker, M. A. Evans-White, and J. T. Scott. Leaf litter stoichiometry is affected by streamwater phosphorus concentrations and litter type. Freshwater Science.

Scott, E. E., M. A. Evans-White, and J. T. Scott. Phosphorus enrichment and light availability differentially affect microbial-mediated leaf litter conditioning.

I. INTRODUCTION

Anthropogenic activities are increasingly disturbing natural environmental regimes including changes to the hydrologic cycle and the biogeochemical processing of carbon (C), nitrogen (N), and phosphorus (P). Rising human population results in increasing conversion of land for agriculture and urban expansion at the expense of environmental integrity. Nutrient pollution involving N and P has become one of the most pervasive threats to aquatic ecosystems worldwide (Carpenter et al. 1998). Eutrophication has largely been considered in the context of autotrophic responses, where effects are in the form of excessive plant and algal growth. Excess nutrient loading into lakes and streams dominated by autotrophic production can result in toxic algal blooms, decreased oxygen concentrations leading to fish kills, and declines in species richness, among other deleterious effects (Smith 2003). Nutrient pollution to low-order, detrital-based streams has received less attention, even though these streams are responsible for a great deal of C, N, and P transformation and transportation, influencing downstream water quality (Fisher and Likens 1973, Mulholland 2004, Alexander et al. 2007).

Low-order, forested streams are characterized as heterotrophic systems where the primary energy input is from the terrestrial biosphere and leaf litter is an important component at the base of the food web (Fisher and Likens 1973, Vannote et al. 1980). Given the poor quality of leaves relative to consumer demand, large resource-consumer elemental imbalances exist which can constrain consumer growth (Sterner and Elser 2002, Cross et al. 2003). However, detritivores are adapted to rely on low quality resources and communities can be negatively impacted by increasing food quality. Many studies have demonstrated relationships between consumer response variables and increasing water column nutrient concentrations (Singer and Battin 2007, Evans-White et al. 2009, Davis et al. 2010). For example, shredder richness

declined across a TP concentration gradient, with a threshold concentration of approximately 50 µg TP/L, presumably as a result of altered detrital stoichiometry and the inability for some species to cope (Boersma and Elser 2006, Evans-White et al. 2009). Leaf litter quality provides the mechanistic link between nutrient enrichment and consumer response, but quantitative changes in litter stoichiometry and the factors controlling altered quality are poorly understood.

Microbial biofilms form on all organic substrates and play a primary role in organic matter (OM) decomposition and nutrient transport and transformation in forested streams (Mulholland 2004, Benstead et al. 2009). Bacteria and fungi are capable of obtaining C and nutrients directly from the leaf as well as from the water column (Suberkropp 1998). Microbial colonization and associated metabolic and nutrient uptake activities serve to alter leaf chemistry, providing a more palatable resource for consumers (Kaushik and Hynes 1971, Cummins 1974, Cross et al. 2003). The magnitude of microbial colonization can depend on several factors including water column nutrient concentrations, leaf species (i.e. inherent chemical characteristics), and light availability.

Nutrient concentrations are typically very low in forested streams and P limitation is common (Elwood et al. 1981, Baldy et al. 2007). Water column nutrient concentrations have been shown to be a great predictor of leaf litter-associated microbial activity (Rosemond et al. 2002, Gulis and Suberkropp 2003, Greenwood et al. 2007, Suberkropp et al. 2010). Increasing nutrient availability can influence decomposition rates (Gulis et al. 2006, Greenwood et al. 2007), microbial biomass accrual (Suberkropp 1995, Rosemond et al. 2002, Benstead et al. 2005) and respiration rates (Stelzer et al. 2003, Greenwood et al. 2007, Suberkropp et al. 2010). The response of microbial activity and decomposition rates to increased nutrient concentrations has been shown to relate to inherent leaf quality characteristics including initial N and P content and

proportions of different C structural compounds (Melillo et al. 1982, Stelzer et al. 2003, Ardón and Pringle 2007, Lecerf and Chauvet 2008). While numerous studies have elucidated the response of microbial activities to various factors controlling leaf litter conditioning, a better understanding of the quantitative change in stoichiometry is needed.

Detritus-based streams have long been considered to be heterotrophic systems where respiration dominates metabolic processes and the presence of a dense riparian canopy results in light limitation of autotrophic production (Vannote et al. 1980, Hill et al. 1995, Franken et al. 2005). However, spatial and temporal variability in canopy cover may result in sufficient light reaching the streambed for algal growth (Rosemond 1994, Sabater et al. 1998, Greenwood and Rosemond 2005). Given the close association of microorganisms within stream biofilms colonizing organic matter, it should be expected that autotrophic production should influence heterotrophic processes (Neely and Wetzel 1997, Rier et al. 2007, Lagrue et al. 2011). In fact, light availability has been shown to influence heterotrophic microbial activity via competition or synergism for nutrient use (Rier and Stevenson 2002, Carr et al. 2005), biofilm redox chemistry (Cole 1982, Neely 1994, Kuhl et al. 1996), and priming effects (Guenet et al. 2010). Therefore, light availability and nutrient enrichment may interact to affect litter processing and stoichiometry.

I conducted two experimental studies with the following objectives: 1) to quantify the response of litter C:P to a range of P concentrations through time and across maple and oak leaves with inherently different quality, 2) to quantify the response of litter C:P to a range of P concentrations in conjunction with altered light regime to simulate shaded versus open canopy systems, and 3) to identify possible mechanisms for this change in litter stoichiometry, including the presence of algae and shifts in microbial metabolism. I hypothesized that 1) litter C:P would

decrease with increasing P availability due to enhanced decomposer activity, 2) this response would be greater for the more labile maple compared to oak leaves due to more available C versus C-limitation, respectively, 3) litter C:P would decrease with increasing P availability due to greater microbial activity, and 4) increased light intensity would result in greater algal biomass and shifts in metabolic regimes, ultimately influencing litter stoichiometry.

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II. LEAF LITTER STOICHIOMETRY IS AFFECTED BY STREAMWATER PHOSPHORUS CONTENT AND LITTER TYPE

A. Introduction

Anthopogenic activities can greatly alter biogeochemical processes involving carbon (C), nitrogen (N), and phosphorus (P). Rising human populations along with increasing agricultural and urban land expansion and intensity result in excess nutrient loading into a myriad of ecosystems (Carpenter et al. 1998, Alexander et al. 2008, Jarvie et al. 2010). Nutrient enrichment of lentic ecosystems has been studied extensively and can result in increased toxic algal blooms, decreased oxygen concentrations, increased turbidity, and declines in species diversity (Smith 2003, Dodds et al. 2009). While the effects on lotic ecosystems are less well understood (Smith et al. 1999, Dodds 2006, 2007), there is a growing body of research demonstrating the effects of nutrient enrichment on leaf litter-associated heterotrophic microbial responses (Greenwood and Rosemond 2005, Dodds 2007, Hill et al. 2011), but more attention to quantitative changes in litter stoichiometry is needed.

Nutrient enrichment to heterotrophic systems can result in increased decomposition and decreased C standing stock (Suberkropp et al. 2010). Low-order streams are often detritus-based systems where the vast majority of the energy available is allochthonous organic matter (Fisher and Likens 1973, Vannote et al. 1980). Leaf litter typically has very poor initial quality (e.g. high C, low N and P) due to its complex carbon structure and the pre-senescent resorption of nutrients (Aerts 1996, Kobe et al. 2005). Immediately upon submersion in streams, microorganisms rapidly colonize leaf surfaces (Suberkropp and Klug 1974). Microbes, especially fungi, perform an essential ecosystem service in streams by transforming leaf litter into a more palatable resource for detritivores (Kaushik and Hynes 1971, Romani et al. 2006,

Gessner et al. 2007). Aquatic fungi are capable of producing extracellular enzymes required to degrade recalcitrant leaf polymers like lignin, and therefore dominate early successional stages of leaf litter colonization (Suberkropp and Klug 1976, Gessner and Chauvet 1994, Romani et al. 2006). Fungi and bacteria obtain carbon and nutrients from the leaves directly and also are capable of obtaining N and P from the water column (Suberkropp and Chauvet 1995, Suberkropp 1998, Findlay 2010). Therefore, heterotrophic decomposers can have a strong influence over water column nutrient concentrations and biogeochemical processes.

Alterations in leaf litter decomposition rates have been reported across natural nutrient gradients (Suberkropp and Chauvet 1995, Rosemond et al. 2002), paired whole-stream enrichment experiments (Elwood 1981, Gulis and Suberkropp 2003, Greenwood et al. 2007), nutrient-diffusing substrate experiments (Robinson and Gessner 2000), and in microecosystem flow-through studies (Howarth and Fisher 1976). Changes in leaf litter decomposition rates are often attributed to enhanced heterotrophic activity in response to nutrient enrichment. Increases in N and P availability can stimulate microbial biomass (Rosemond et al. 2002, Benstead et al. 2005, Suberkropp et al. 2010) and respiration rates (Elwood et al. 1981, Stelzer et al. 2003, Suberkropp et al. 2010) associated with leaf litter. But, relatively few studies have demonstrated how increased nutrient availability may affect leaf litter quality (as defined by nutrient content and stoichiometric ratios). Elwood (1981) enriched an oligotrophic stream (background SRP and DIN were 4 and 35 µg/L, respectively) with either 60 or 450 µg/L SRP and measured an 83% increase in P content of red oak leaves in the enriched reaches compared to the control. Cross et al. (2003) and Small and Pringle (2010) also reported increases in %P and subsequent decreases in C:P of leaf litter in streams with greater N and/or P availability, but more data are needed to understand the magnitude of change caused by nutrient enrichment in diverse stream ecosystems.

Understanding this quantitative link between water column P availability and leaf litter stoichiometry can be important for predicting ecosystem changes resulting from anthropogenic nutrient enrichment of streams.

Phosphorus enrichment can alter leaf litter P content and the C:P ratio, leading to decreased resource-consumer imbalance (Cross et al. 2003). This difference in elemental composition between resource and consumer can limit consumer productivity and provide an important selection pressure to promote species diversity in streams (Dodds et al. 2009, Evans-White et al. 2009). Although some studies have explored the effects of variable water column nutrient availability on leaf litter-associated microbial biomass and decomposition (Gulis and Suberkropp 2003, Baldy et al. 2007), and detrital stoichiometry on the trophic response of invertebrates (Hladyz et al. 2009, Small et al. 2011), broad assumptions are often made concerning the chemical alterations in the basal food resources. Specifically, many studies have qualitatively described an increase in litter P content as inferred by greater microbial biomass, while few have explicitly quantified changes in litter stoichiometry (Abelho and Graça 2006, Webster et al. 2009, Cheever et al. 2012). Additionally, while much experimental manipulation has been done with N or N+P, very few studies have focused on P explicitly. This is an important avenue for research given common P-limitation in forested streams (Elwood et al. 1981, Chadwick and Huryn 2005, Ardón and Pringle 2007).

The objective of this study was to explore the effect of P enrichment on leaf litter stoichiometry using laboratory experimentation. We were interested in quantifying the effect of P exposure time and concentration on leaf litter chemistry and exploring response differences between post oak (*Quercus stellata* Wangenh.) and sugar maple (*Acer saccharum* Marsh.). These are common in many deciduous forests throughout the USA and are among the dominant

species in the study region. Additionally, post oak and sugar maple have inherently different chemical properties (specifically in structural C compounds). Leaf litter is primarily a pulsed organic carbon input and time-series changes to quality might variably affect different shredder taxa. We predicted that the C:P ratio would decrease through time and with increasing levels of P enrichment for both leaf types. Additionally, given similar initial N and P content across maple and oak leaves but greater lignin content in oak, we predicted that P enrichment of the labile maple leaves would elicit a greater response from microorganisms compared to that of the more recalcitrant oak because microorganisms on oak leaves would be more constrained by C availability.

B. Methods

A laboratory microcosm experiment was conducted using a factorial design to examine the effect of leaf litter species (sugar maple and post oak) and increased soluble reactive P concentration (SRP additions of 0, 50, or 500 µg / L) on litter stoichiometry. Three replicate microcosms were used for each of the P treatments and both leaf types, separated into different leaf bags, shared each microcosm. These microcosms were sampled over time (day 0, 5, 8, 13, 20, 28, 36, 43, 59, 72, 95, 115, and 139) for litter C and P content. Maple and oak leaves were collected in the southwestern Ozark Highlands shortly after abscission in November 2010, dried at ambient temperatures for 2 weeks, cut into 13.5 mm diameter leaf disks with major veins avoided, and stored at 40°C for 2 weeks until the start of the experiment. Approximately 40 disks of each species were placed into separate 10-mm mesh bags so that a total of 9 bags for each leaf species were filled. One-liter microcosms were filled with 750 mL unfiltered stream water from Jones Creek, a third-order stream near Winfrey, Arkansas, USA, that has low

concentrations of SRP (< 6 μg/L) and moderate concentrations of nitrate (NO_3-N, 345 ± 32 μg/L). Therefore, with increasing P enrichment, ambient molar N:P decreased: 130, 14, and 1.5 for the control, low-P, and high-P treatments, respectively. In streams polluted with P, particulate P can be a large proportion of the increasingly available pool, but streams receiving large volumes of treated waste-water effluent can have very elevated SRP. We used a high SRP enrichment (500 μg/L) to simulate high P enriched conditions. Two leaf bags, one containing each leaf species, were placed into individual microcosms. All microcosms were constantly aerated and were flushed on each sample day with fresh, unfiltered stream water plus appropriate SRP amendments. In between each of the last three sample days, microcosms were topped off with tap water (approximately 200 mL maximum) to maintain a constant volume. Tap water concentrations for SRP and NH_4-N were below detection, but NO_3-N was1300 μg/L.

Two leaf disks of each leaf species were collected from microcosms on each sampling day and dried for approximately 48 hours at 50°C, then frozen for later analysis of organic C (litter C) and phosphorus (litter P). Frozen disks were re-dried for 24-48 h (50°C) and analyzed for C content using a Thermo Flash 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Netherlands). Litter P was measured by ashing disks at 550 °C and then oxidizing P by perfulfate digestion before analyzing colorimetrically using the ascorbic acid method (APHA 2007). All ratios are reported in molar units.

Statistical Analyses

To evaluate the stoichiometric saturation of P in litter ($C:P_{sat}$) and the time required to elicit such a response in the enrichment experiment, we used a Michaelis-Menten kinetic model in SigmaPlot 12.0 for all replicates (Systat Software, Inc., San Jose, CA). This allowed us to determine the saturating P:C (inverted for positive response with time) ratio and the amount of

time required for this to occur. Since the C:P in the control treatments for both maple and oak did not change through time, C:P values from all sample days were used to calculate respective means. Carbon:P saturation and time to saturation data were analyzed using one-way analysis of variance (ANOVA) to quantify how these parameters were influenced by leaf species and enrichment concentration. Leaf N:P data were analyzed with a one-way ANOVA using all data after 8 d (before this time, chemistry within a treatment was too variable and N:P did not significantly change through time). Percent C also was analyzed with a one-way ANOVA using all data after 8 d. Specifically, we used PROC GLM in SAS (version 9.1, SAS institute, Cary, North Carolina) to conduct the ANOVAs and used the REGWQ multiple range analysis to test for differences among individual treatments when the omnibus *F* test was significant at alpha = 0.05.

C. Results

Leaf litter %P increased through time in the low- and high-P treatments, and differed across P treatments, from 0.05 to 0.25 and 0.05 to 0.15 for maple and oak, respectively (Fig. 1). This led to a subsequent decrease in leaf C:P from 2500 to 500 and from 2000 to 1000 for maple and oak, respectively (Fig. 2). Carbon:P saturation ($C:P_{sat}$) ratios were significantly different across P enrichment levels and leaf species (Table 1). For all but the control treatments, maple leaf $C:P_{sat}$ decreased significantly more than oak leaf $C:P_{sat}$ for a given P concentration (Table 1). Specifically, maple leaf $C:P_{sat}$ decreased to approximately 490 and 300 for low-P and high-P treatments, respectively, and were not significantly different from each other. Oak leaf $C:P_{sat}$ decreased to approximately 1450 and 790 for low-P and high-P treatments, respectively, and were significantly different from each other. High-P enrichment of oak leaves elicited a similar

response to low-P enrichment of maple leaves. Maple and oak leaf C:P in the control treatments were similar to each other (C:P approximately 2550) and did not change through time.

Maple leaf chemistry saturated later in time than did oak leaf chemistry (approximately 135 and 30 d, respectively), and there was no effect of P concentration on time to saturation for either species (Table 1). The change in litter C:P was primarily a result of changes in %P (Fig. 1) rather than %C, which ranged from 42-51% and generally increased slightly through time for maple treatments only ($p < 0.01$). Leaf litter mean N:P ranged from 25 to 70 and was significantly related to leaf species and SRP, following an almost identical statistical pattern to C:P (Table 1). Leaf litter %N and C:N did not differ across treatments.

D. Discussion

The objective of this study was to quantify the effect of P enrichment on stream leaf litter stoichiometry. In addition to the simple quantitative link between stream P and litter stoichiometry, we were specifically interested in whether P enrichment would differentially affect litter stoichiometry depending on leaf litter species, and if the timing of litter C:P saturation would vary with leaf type and P enrichment level. Indeed, experimental results showed that P enrichment affected the C:P saturation of litter differently depending on litter type, and that the timing of C:P saturation also varied with litter type.

1. *Effect of P enrichment on leaf litter stoichiometry*

Leaf litter %P, C:P, and N:P exhibited significant differences across experimental P treatments and leaf species. Percent P increased with experimental P concentration and led to decreases in C:P and N:P for both leaf species. Microbial activity can lead to increased leaf litter

nutrient content as in-stream conditioning progresses (Kaushik and Hynes 1971). In undisturbed forested stream systems, biotic activity is often limited by N or P (Elwood et al. 1981, Hladyz et al. 2009, Hill et al. 2010). Phosphorus enrichment of streams can result in increased microbial activity including enhanced production and respiration rates (Rosemond et al. 2002, Gulis and Superkropp 2003, Greenwood et al. 2007, Suberkropp et al. 2010). Consistent with other studies, enriched leaves in this study had significantly higher P content and consequent decreases in C:P and N:P compared to controls, suggesting that microbes may have been P-limited (ambient stream molar NO_3^--N:SRP was approximately 130).

Leaves exposed to high-P enrichment increased in % P by as much as 30 and 350% for oak and maple, respectively. Other studies have reported an approximately 60-80% increase in litter %P with P enrichment relative to controls (Elwood et al. 1981, Cross et al. 2003). The relatively large change in maple C:P could have been a function of our experimental design, but other indicators confirm that this change may not be unreasonable. For example, the range of C:P ratios of maple leaves was 500-3500, changing with time and treatment, and the range in C:P ratios across the P gradient of Ozark streams was from 1000 – 3000 (Scott et al. in press). Stelzer et al. (2003) reported an initial %P and C:P of sugar maple leaves of 0.049% and 2412, respectively, very close to that measured in the current study. Small and Pringle (2010) reported C:P of mixed litter in tropical streams from 500-2000, depending on stream water P. Similarly, Cross et al. (2003) reported mean C:P of mixed leaf litter ranging from 3000 in an enriched reach to 5000 in the reference reach. Thus, the range of C:P observed in our experimental manipulations was not out of the ordinary.

The magnitude of P enrichment can also influence the leaf litter stoichiometric response differentially across leaf species. Our experimental study was intended to simulate potential

extremes of P concentrations in streams, including those receiving treated wastewater. The high P concentrations (500 µg SRP/L) did not yield a significantly lower C:P for maple litter compared to concentrations of approximately 50 µg/L, but did for oak litter (Table 1, Fig.3). Additionally, litter C:P ratios were similar for maple and oak when no P was added. These data may allude to a potential threshold in ambient P concentrations where litter C:P may not change with higher concentrations of P enrichment, and this threshold may vary depending on leaf species (Fig. 3). Other studies have indicated that this threshold may occur between 25-50 µg/L P (Rosemond et al. 2002, Small and Pringle 2010). This is important because macroinvertebrate richness has been shown to respond to changes in resource quality (Evans-White et al. 2009). While further study is necessary to fill in the P concentration gaps between approximately 60 and 500 µg/L, these results provide a strong potential for use in establishing nutrient criteria in streams with detritus-based food webs.

One potential limitation of our experimental design was that the microcosms lacked flow through to constantly replenish nutrient supply. Thus, the experimental units could have experienced an extreme drawdown of ambient nutrients that may have resulted in more severe nutrient limitation, especially in the control and low-P treatments. We tested this hypothesis in association with an ongoing study. We found that while ambient concentrations of SRP quickly decreased to below detection after beakers were flushed, mean water column TP concentrations were approximately 45 and 65 µg/L in the control and low-P treatments, respectively. This suggests that P turnover from the water column was probably an important P source to litter, which is similar to conditions often observed in streams (Dodds 2003).

2. *The role of C lability in detrital stoichiometry*

The response of maple leaves was significantly different from that of oak, with maple leaves responding earlier, longer, and to a greater extent to P enrichment. Both maple and oak leaves had significantly different C:P_{sat} ratios on the low P treatment compared to their respective controls. However, maple leaf C:P_{sat} from the low- and high-P treatments were not different from each other, but oak leaf C:P_{sat} from low- and high-P treatments were different (Table 1). This suggests that an SRP concentration of 50 μg /L saturated microbial P uptake on the maple leaves but not on the oak leaves. We also found that low-P enrichment of maple leaves yielded a response in litter C:P similar in magnitude to high-P enrichment of oak leaves. Therefore, microbially-mediated litter stoichiometry depends not only on stream water P concentrations, but also on the intrinsic qualities unique to the leaf species.

Fungal activity and decomposition can be regulated by inherent litter quality such as lignin content (Gessner and Chauvet 1994, Gessner et al. 2007). Ardón and Pringle (2007) demonstrated that the availability of labile C can influence the stimulatory effect of P-enrichment by experimentally enriching low-lignin (*Trema integerrima*) and high-lignin (*Zygia longifolia*) leaf species. They found that biofilm respiration was C-limited on high-lignin *Zygia* and thus P enrichment had no stimulatory effect, while respiration was not C-limited on low-lignin *Trema* and P enrichment did exhibit a stimulatory effect. Our study indicated that the relatively labile maple leaves were more sensitive to P enrichment than the relatively recalcitrant oak leaves. Maple leaf C:P was equal at low- and high-P concentrations, while oak leaf C:P was different among the two P enrichment levels. However, other studies have shown opposite effects. For example, Greenwood et al. (2007) reported that N+P enrichment stimulated relatively recalcitrant rhododendron leaves compared with relatively labile red maple leaves. They

assumed relative lability based on variation in initial leaf C:N (high rhododendron, low red maple). Initial N and P content of oak and maple leaves from our study were similar, but these leaf taxa are known to differ in the amount and types of structural C compounds such as lignin content (Melillo et al. 1982, Hladyz et al. 2009). Absolute lignin content and lignin:P have been shown to be the most important predictors and drivers of leaf litter-associated microbial activity (Gessner and Chauvet 1994, Hladyz et al. 2009), which is supported by our results.

3. *Timing of litter conditioning*

One of the major assumptions about experimental stream enrichment is the time required for microorganisms to induce chemical change in leaf litter. Ardón and Pringle (2007) reported increased respiration in response to P enrichment by the more labile *Trema integerrima* but not by the more recalcitrant *Zygia longifolia* leaves over a 16 d period. This time (16 d) may have been too short to capture a stoichiometric change in a recalcitrant leaf species. By the end of our experiment, higher quality maple leaves had significantly greater P content and lower C:P than the more recalcitrant oak leaves. Michaelis-Menten kinetics on litter P:C revealed that microbial activity stabilized the stoichiometric composition of leaves at approximately d 135 and 32 for maple and oak leaves, respectively.

Our experiment was designed to demonstrate microbial-mediated P immobilization from the water column. Under certain circumstances, leaf litter may function as a net source of nutrients back into the water column (Webster et al. 2009). We acknowledge that laboratory studies may not be well extrapolated to natural systems. However, these experiments can be used to elucidate general trends in the potential leaf litter-associated microbial response to varying water column P concentrations and N:P ratios of available nutrients. Further study is

needed to refine our understanding of potential mechanisms involved that may cause variation in the microbial and stoichiometric response to enrichment.

4. *Conclusions*

Although it is well-known that microbial activity can be enhanced with increasing nutrient availability, few studies have looked quantitatively at the resulting elemental changes of the leaf litter, particularly with detailed time-series measurements. We found that increasing the availability of P led to increased leaf litter quality by decreasing the C:P ratio. Additionally, we used several P concentrations which may lead to enhanced understanding of possible threshold and saturating concentrations. Our work has shown that the stoichiometric response of leaf litter was dependent upon leaf species, level of P enrichment, and time of incubation. Furthermore, the dominant species of riparian cover may affect the response of leaf litter decomposition and C storage, potentially altering the trophic base of head-water stream foodwebs. Understanding this quantitative link between riparian community structure, water column P availability and leaf litter stoichiometry could inform management decisions for riparian zones, as well as nutrient criteria in streams.

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G. Tables

Table 1. Results from ANOVAs comparing effect of phosphorus concentration and leaf type on the N:P ratio ($F_{5, 179} = 50.4$), the saturation C:P ($F_{5,12} = 115.6$, $p < 0.0001$) and day of C:P saturation ($F_{3,8} = 9.28$, $p < 0.01$) of leaf litter based on the Michaelis-Menten saturation models. Values are means ± SD. Treatments and/or leaf types with the same letters were not statistically different. The R^2 values indicate the strength of the Michaelis-Menten model when all replicates for each treatment were combined. An asterisk (*) denotes treatments that could not be modeled with a saturation curve and the C:P value is the mean across all sampling days.

Treatment		**P addition (µg/L)**	**N:P**		**C:P$_{sat}$**		**Day of C:P$_{sat}$**		**Adj. R^2**
Maple	Control*	0	63 ± 22	A	2573 ± 271	A			
	Low P	50	31 ± 8	CD	489 ± 57	CD	129 ± 35	A	0.77
	High P	500	24 ± 9	D	298 ± 54	D	142 ± 53	A	0.80
Oak	Control*	0	68 ± 19	A	2542 ± 237	A			
	Low P	50	44 ± 6	B	1453 ± 95	B	16 ± 4	B	0.57
	High P	500	36 ± 10	BC	792 ± 116	C	48 ± 30	B	0.42

H. Figure legends

Figure 1. Scatter plot of laboratory experiment % phosphorus (P) of (a) maple and (b) oak leaf litter through time. White circles represent zero added P, black circles represent the low-P treatment (50 µg PO_4^{3-}– P/L) and black triangles represent the high-P treatment (500 µg PO_4^{3-}– P/L). Error bars represent ± 1 SD.

Figure 2. Scatter plot of laboratory experiment carbon:phosphorus (C:P) molar ratio of (a) maple and (b) oak leaf litter through time. White circles represent zero added P, black circles represent the low-P treatment (50 µg PO_4^{3-}– P/L) and black triangles represent the high-P treatment (500 µg PO_4^{3-}– P/L). Error bars represent ± 1 SD.

Figure 3. Scatter plot of mean carbon:phosphorus saturation values ($C:P_{sat}$) across SRP concentrations for maple (white circles) and oak (black circles). These data allude to a potential threshold concentration where litter chemistry no longer changes with increasing nutrients, and that this threshold may differ among leaf species.

I. Figures

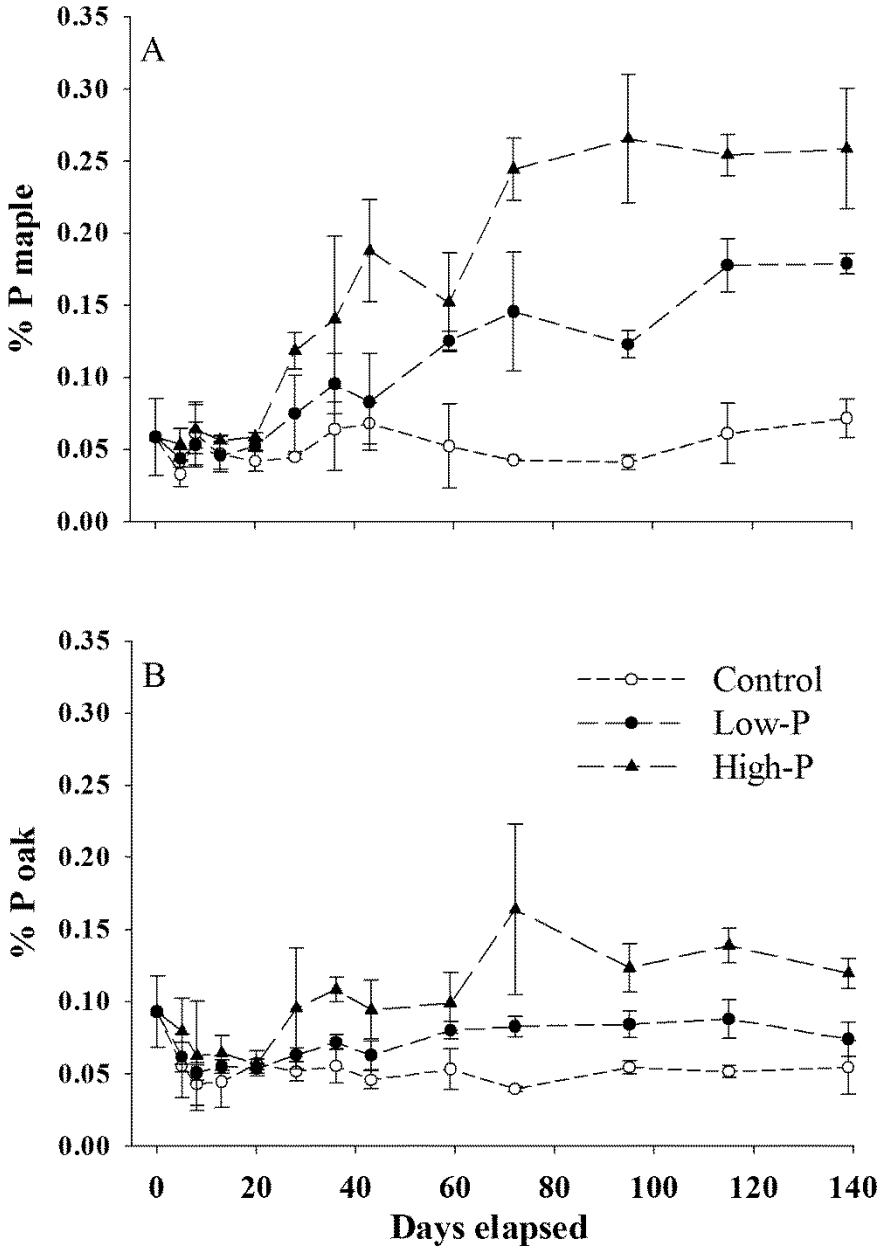

Figure 1

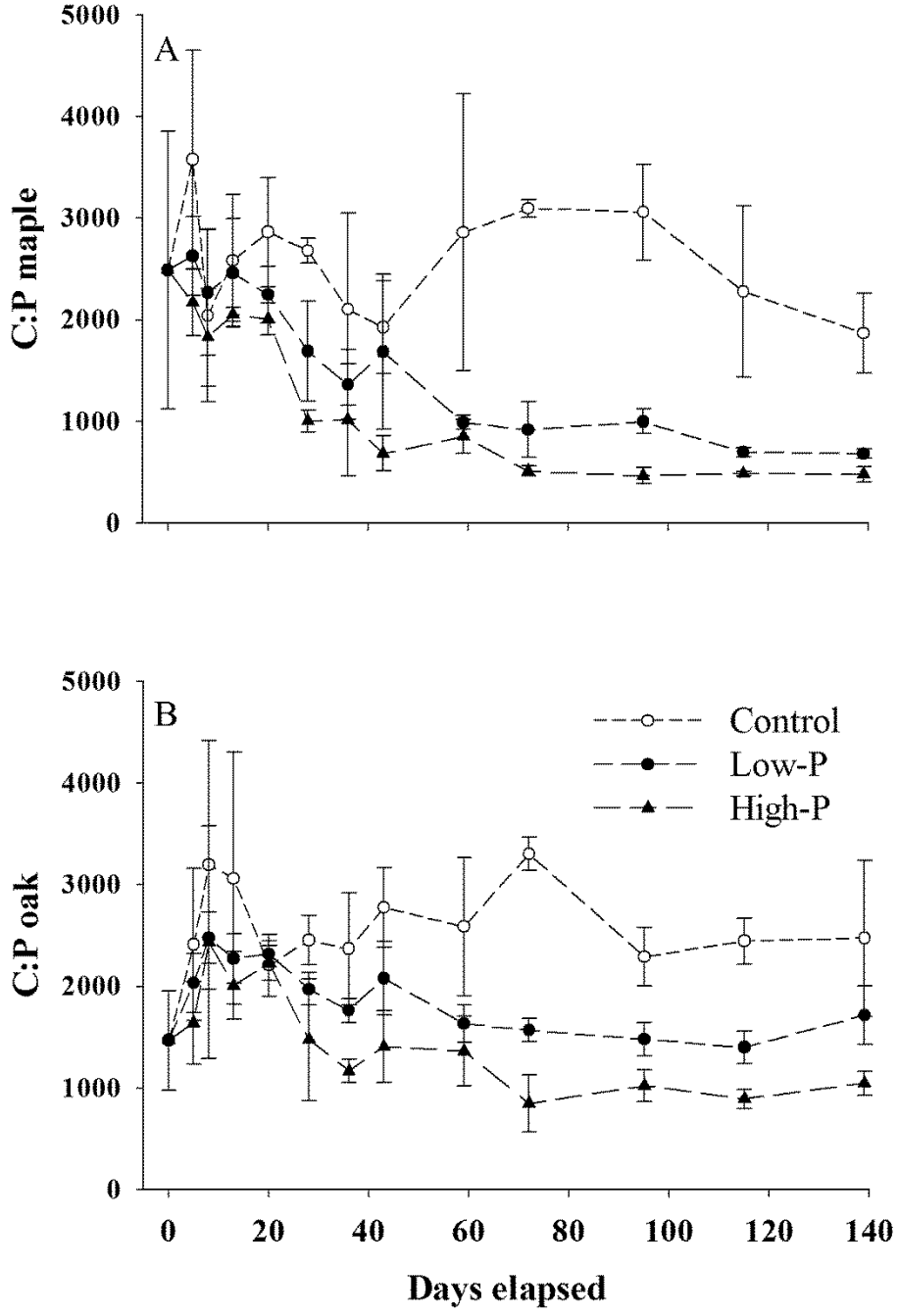
A
B
C:P maple
C:P oak
Control
Low-P
High-P
0
1000
2000
3000
4000
5000
0
20
40
60
80
100
120
140
Days elapsed

Figure 2

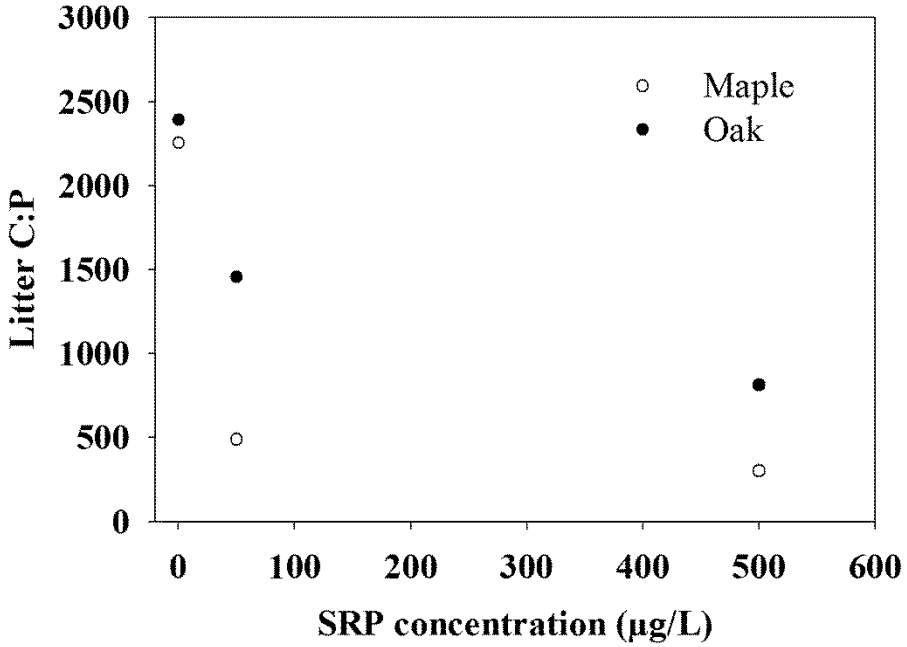
3000
2500
2000
1500
1000
500
0
Litter C:P
0
100
200
300
400
500
600
SRP concentration (µg/L)
Maple
Oak

Figure 3

J. Appendix

I hereby state that Erin E. Scott was the lead author on chapter 2 of this thesis titled “Leaf litter stoichiometry is affected by streamwater phosphorus content and litter type” and that she completed at least 51% of this work.

J. Thad Scott
Thesis Director

III. PHOSPHORUS CONCENTRATION AND LIGHT AVAILABILITY DIFFERENTIALLY AFFECT MICROBIAL-MEDIATED LEAF LITTER CONDITIONING

A. Introduction

Anthropogenic nutrient pollution is a pervasive threat to aquatic ecosystems worldwide. Nutrient enrichment can greatly influence biogeochemical processes such as primary production, decomposition, and nutrient spiraling in streams, thus impacting ecosystem structure and function. Ecological stoichiometry theory suggests that nutrient cycling can be driven by nutrient availability relative to organism demand and can be used to make predictions about how altered nutrient regimes might impact C and nutrient dynamics in stream ecosystems (Elser et al. 1996, Sterner and Elser 2002, Evans-White et al. 2009). Effects of increased nutrient loading into detrital systems are still poorly understood, especially in the context of altered litter quality and stoichiometry.

Microbial biofilms develop on organic substrates and play a critical role in mediating C and nutrient transport and transformation in detrital systems (Mulholland 2004, Benstead et al. 2009). These microbial decomposer communities immediately colonize leaves with poor carbon-quality and are integral in the immobilization and mineralization of C, N, and P in streams (Barlocher and Kendrick 1974, Webster et al. 2009). In temperate streams, litter inputs are pulsed during autumn and an immediate increase in nutrient uptake occurs (Mulholland 2004). But, leaves might also act as a net source of nutrients to the water column during later stages of decomposition (Webster et al. 2009). Microbial activity on leaves results in a more palatable food resource for primary consumers and thus forms an important link between nutrient demand from stream water and consumer production (Cummins 1974, Barlocher 1985, Graça 2001).

Streams are becoming increasingly modified by anthropogenic activities which can influence microbial-mediated leaf litter processing. Forested streams are often characterized by low water column nutrient concentrations and as such often exhibit N and/or P limitation (Elwood et al. 1981, Baldy et al. 2007). Nutrient enrichment has been shown to increase decomposition rates (Gulis et al. 2006, Greenwood et al. 2007) leading to decreased C standing stocks (Suberkropp et al. 2010). Higher rates of decomposition have been attributed to enhanced heterotrophic microbial activity, such as increased biomass accrual (Suberkropp 1995, Rosemond et al. 2002, Benstead et al. 2005) and respiration rates (Stelzer et al. 2003, Greenwood et al. 2007, Suberkropp et al. 2010). But, few studies have considered how changes in light availability might interact with nutrient enrichment to affect ecosystem processes like litter conditioning and metabolism (Sabater et al. 2005).

Low order forested streams are characterized as being predominantly heterotrophic with the primary inputs of C and energy originating from the terrestrial biosphere (Fisher and Likens 1973, Vannote et al. 1980). Additionally, the presence of dense riparian cover generally results in light limitation of autotrophic production (Hill et al. 1995, Franken et al. 2005, Greenwood and Rosemond 2005). However, there may be spatial or temporal instances where light can reach the stream bed (Rosemond 1994, Sabater et al. 2011) and stimulate autotrophic activity on organic substrates (Golladay and Sinsabaugh 1991, Sabater et al. 1998). Light availability has been shown to influence heterotrophic microbial activity via the close interaction with algae (Neely 1994, Neely and Wetzel 1997, Rier et al. 2007, Lagrue et al. 2011). These microbial interactions can include competition or synergism for the use of N and P (Rier and Stevenson 2002, Carr et al. 2005). Also, algal photosynthesis can provide oxygen to fuel heterotrophic metabolism (Neely 1994, Kuhl et al. 1996) while heterotrophic respiration can supply CO_2 for

algal production (Cole 1982). Bacteria and fungi can further benefit from algal-derived labile C and use algae as a substrate for colonization (Rier and Stevenson 2002). Additionally, priming effects occur when the addition of labile organic C (e.g. from algal exudates) enhances decomposition of recalcitrant organic matter like leaves (Guenet et al. 2010). Carbon and nutrient availability are likely important factors influencing the priming effect (Guenet et al. 2010). Algal growth on detritus also can have important implications for consumers by directly affecting food quality (Cross et al. 2005, Franken et al. 2005). Given the potential for close association between heterotrophic and autotrophic microorganisms, an interactive effect of nutrient enrichment and light availability on litter processing should be expected.

The effects of increased nutrient concentrations and light availability on stream detrital processing are poorly understood. It was our objective to explore the interactive effect of P enrichment and light availability on microbial-mediated leaf litter conditioning, specifically in regards to shifts in metabolic regimes and stoichiometric changes. A laboratory study using 3 phosphorus concentrations across 2 light levels was conducted to test the effect that P enrichment and algal activity might have on C and P dynamics associated with decomposing maple leaves. We measured algal biomass, microbial respiration and net metabolic rates, P uptake and release rates, and we quantified changes in litter C:P. We hypothesized that C:P would decrease to a constant minimum through time and that this response would be greater with increasing levels of P enrichment due to enhanced microbial activity (e.g. respiration and P uptake rates). Additionally, we hypothesized that algal biomass would increase with increasing light and P availability and that this would influence metabolic regimes, ultimately influencing litter elemental ratios. Finally, we expected to measure net P uptake during early stages of decomposition with a shift to net P release during later stages.

B. Methods

1. *Experimental design*

A laboratory microcosm experiment was conducted using a factorial design to examine the effect of dissolved P concentration (additions of 0, 50, or 500 µg SRP/L; "low-", "moderate-", and "high-P", respectively) and light availability (15 or 500 µmol m^{-2} s^{-1}; "low" and "high" light, respectively) on litter stoichiometry. Maple leaves were collected in the southwestern Ozark Highlands shortly after abscission in autumn 2010 and stored at room temperature until use. Leaves were wetted and 13.5-mm diameter leaf disks were cut with major veins avoided and stored at 40°C for three weeks until the start of the experiment. Approximately 120 disks were placed into each 1-L microcosm which were filled with 750 mL screened (50-µm mesh to exclude all macroinvertebrates) stream water collected from Jones Creek, a third-order stream near Winfrey, Arkansas, USA, that had relatively low mean concentrations of SRP (10.6 µg/L) and DIN (527 µg/L) during the study period.

Three replicate microcosms were used for each of the 6 factorially-crossed treatments. These microcosms were sampled over time (day 0, 3, 8, 15, 22, 29, 36, 43, 57, 71, 92, 113, and 134) for litter C and P content, algal biomass, microbial metabolism (respiration and net oxygen flux), and P uptake and release rates. All microcosms were amended with 1 mg/L NO_3^--N and with SRP according to the concentrations described above. These amendments resulted in water column molar N:P of approximately 338, 56, and 6 for the low-, moderate-, and high-P treatments, respectively. Microcosms were incubated in a water bath at a constant temperature of 10°C and constantly aerated. Low and high light treatments were separated by an opaque barrier and light regimes were kept at 14:10 dark:light cycles. Each week, microcosms were

flushed with fresh stream water and appropriate N and P amendments. We also collected an additional 5 L of stream water before each sample date for use in metabolism and ^{33}P assays.

2. *Litter stoichiometry*

Four leaf disks were collected from microcosms on each sampling day, dried for approximately 48 h at 50°C, and frozen until analysis of organic (litter) C and P. Frozen disks were re-dried for 24-48 h at 50°C, homogenized into a fine powder using a Cresent Wig-L-Bug **(**Crescent Dental Manufacturing, Elgin, IL**)**, re-dried, and analyzed for C and N content using a Thermo Flash 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Netherlands). Litter P content was measured by ashing leaf powder at 550 °C for 4 hours followed by oxidation of P by persulfate digestion and then analyzing colorimetrically using the ascorbic acid method (APHA 2007). All ratios are reported in molar units.

3. *Algal biomass and microbial metabolism*

Leaf litter-associated algal biomass was estimated by measuring chlorophyll *a* concentrations on leaf disks using a Turner Designs Trilogy Laboratory Fluorometer (Sunnyvale, CA) following extraction with 90% acetone in the dark at 4°C for 24 h. Chlorophyll *a* was quantified fluorometrically (665 nm) and corrected for phaeophytin by acidification. Microbial metabolism (respiration and net production) was estimated as the change in dissolved O_2 concentration through time using a Membrane Inlet Mass Spectrometer (MIMS; Kana et al. 2004). This method analyzes gaseous O_2 to argon ratios using a MIMS equipped with a Pfeiffer Prisma mass spectrometer and a Bay Instruments DGA membrane inlet S-25-75. Two leaf disks from each microcosm were sampled for respiration (dark) and net metabolism (light). Disks

were immediately placed in chambers (24 mL) and filled with screened (50-µm) stream water from Jones Creek that was amended with N and P at the previously described concentrations for each P treatment. Vials were capped and sealed with no headspace and incubated for approximately 14-20 hours at 10°C under designated light conditions (i.e. low or high light) and in dark conditions. Vials with amended stream water only (no leaf disk) were used as controls and to measure initial concentrations for each P treatment. Vials for initial O_2 concentrations were immediately preserved with ZnCl. After oxygen concentrations were measured, leaves were dried, weighed, ashed at 550° C for 4 hours, and then re-weighed to determine ash free dry mass (AFDM). Respiration and NPP rates are reported in µg O_2 mg $AFDM^{-1}$ h^{-1}. Respiration rates were translated to cm^{-2} by dividing oxygen flux by leaf area rather than AFDM and were then calculated in terms of C cm^{-2} h^{-1} according to methods from Wetzel and Likens (2000). Briefly,

$$R_C = (R_O)(R_Q)(0.375) \quad \text{(Eq. 1.1)}$$

where R_C is the respiration rate in µg C cm^{-2} h^{-1}, R_O is the respiration rate in µg O_2 cm^{-2} h^{-1}, R_Q is the respiratory quotient, assumed to equal 1.0, and 0.375 is the ratio of moles of C to moles of O_2. Respiration values were further modified to estimate C assimilation rates for the treatments where O_2 flux indicated net heterotrophy. Carbon assimilation rates were calculated as

$$A = \frac{(MGE)(R)}{(1-MGE)} \quad \text{(Eq. 1.2)}$$

where A is the C assimilation rate in µg C cm^{-2} h^{-1}, MGE is microbial growth efficiency assumed to equal 0.6, and R is the respiration rate in µg C cm^{-2} h^{-1}. A MGE of 0.6 was used because microbes in our study were likely minimally stressed and thus efficiency was likely high (Keiblinger et al. 2010). Net primary production (NPP) rates of high light treatments were calculated in terms of C cm^{-2} h^{-1} according to Wetzel and Likens (2000):

$$NPP_C = \frac{(NPP_O)(0.375)}{P_Q} \quad \text{(Eq. 1.3)}$$

where NPP_C is the NPP rate in µg C cm^{-2} h^{-1}, NPP_O is the NPP rate in µg O_2 cm^{-2} h^{-1}, P_Q is the photosynthetic quotient, assumed to equal 1.2, and 0.375 is the ratio of moles of C to moles of O_2.

4. *Phosphorus uptake and mineralization*

Phosphorus uptake and release rates were measured using a ^{33}P (as PO_4-P) radiotracer. Leaf disks were incubated in 10 mL screened (50-µm) stream water from Jones Creek with a small amount of added unlabeled SRP (3.3 µg/L) and 0.125 µCi of ^{33}P. To correct for abiotic uptake of P, some vials were prepared with 4 mL of 10% formalin and 6 mL filtered stream water with the same amount of added unlabeled and labeled P as in sample vials. Leaves were incubated for 2 hours to allow for uptake of P and then transferred to new vials filled in the same manner as before, but without the addition of labeled P, in order to measure mineralization.

Incubations for mineralization were conducted over a 10 h period. From each mineralization vial, 250 µL aliquots of water were taken at 2, 4, 6, 8, and 10 h, and transferred to 6-mL scintillation vials with 5 mL of scintillation fluid added. An extended mineralization assay was run for 5 days in order to be sure that mineralization was captured. In the extended mineralization assay, 250 µL aliquots were collected at 2, 10, 24, 48, 72, 96, and 120 h, and transferred to 6-mL scintillation vials with 5 mL of scintillation fluid added. It was learned in hindsight that the longer mineralization incubation was ideal (Harrison 1983, Hauer and Lamberti 2007). However, results demonstrate that at both incubation durations, mineralized P was only a small proportion of total P immobilized.

At the end of the mineralization incubation, leaf disks were transferred to 6-mL scintillation vials with 300 µL of tissue solubilizer and incubated in a water bath for 16 h at 45°C. Vials were removed from the water bath and 5 mL of scintillation fluid was added. All liquid and leaf radioactive samples for P uptake and mineralization methods were allowed to settle for 3 days and then liquid scintillation analyses were performed with a Beckman model 6500 multi-purpose scintillation counter (Beckman Coulter, Inc.). Separate quench curves for leaf and liquid samples were prepared to convert radioactivity in counts per minute (CPM) to disintegrations per minute (DPM). The H number was used as a proxy for quench and was correlated with the ratio of CPM to expected DPM. Linear regression analysis was used and provided a good fit for the prediction of DPM for leaf samples (equation: $y = -0.0019x + 1.0566$, $r^2 = 0.95$, $p < 0.0001$). For liquid samples, the range in H numbers was small and quench was relatively constant ($p > 0.1$), so the average quench ratio (0.81, SD 0.02) was used to apply a correction factor to all samples. All values were corrected for decay and abiotic uptake. Radioactive P was extrapolated to total P using the proportion of labeled P to total P (stream SRP + added unlabeled SRP + added ^{33}P labeled SRP). We expected to estimate mineralization rate based off the slope of the regression lines. But, because measured mineralized P was highly variable and followed no trend throughout the incubation, data could not be modeled using regression analysis. Therefore, we used the maximum P release value (e.g. sampled aliquot) for a given incubation time to make a conservative mineralization estimate. Phosphorus uptake rates were calculated by adding the maximum release P to leaf P. Phosphorus uptake rates are reported in ng P cm^{-2} h^{-1}.

5. *Dissolved nutrients*

Water samples were collected each week, filtered using a 0.7-µm filter (Whatman GF/F), and stored at 4°C until being analyzed for SRP, NO_3^- + NO_2^- –N, NH_4^+ - N, and dissolved organic carbon (DOC). Samples for SRP were analyzed using the ascorbic acid method. The cadmium reduction method and the salicylate method were used for NO_3^- + NO_2^- –N and NH_4^+ - N analyses, respectively. DOC was analyzed using a Shimadzu TOC-V CSH (Shimadzu Scientific Instruments, Columbia, MD). Light levels were measured weekly with Licor quantum sensor (LI-250A Light Meter/Photometer, LI-193 bulb, LI-COR®, Lincoln, NE) just above the water surface.

6. *Statistical Analyses*

All statistical analyses were conducted using SAS 9.3 (SAS institute, Cary, North Carolina) and SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA). To test our prediction that C:P would decline to a constant minimum ($C:P_{min}$), we ran a one-way ANOVA for each treatment with time as the independent variable. To evaluate differences in $C:P_{min}$ among treatments, we used a one-way ANOVA with all treatments included beginning with C:P values on d 36, given results of the statistical analysis for $C:P_{min}$. To evaluate the time required for P and light treatments to affect litter stoichiometry, we ran a repeated-measures analysis of variance (rm ANOVA) with univariate analysis, which revealed significant changes beginning on d 29. Repeated-measures ANOVAs were also used to analyze the effect of time and treatment for chlorophyll *a*, respiration rates, net metabolic rates, and P cycling metrics. When rm ANOVAs were significant, we used one-way ANOVAs to identify those treatments that were different. Because we wanted to compare across treatments for chlorophyll *a*, respiration, and

net metabolism in the context of changes in litter C:P, we ran one-way ANOVAs for these parameters using data beginning with d 29. The relationship between C:P and net O_2 flux was analyzed using regression analysis. Regression analysis was also used to determine the slopes of the lines for C assimilation rates (for low light treatments) and net primary production (for high light treatments) versus P uptake rates. Analyses of covariance were used to test if the slopes for C assimilation and NPP versus P uptake were similar. Slopes were considered equivalent to the C:P of nutrient assimilation ($C:P_{assimilated}$) for each treatment.

C. Results

Leaf litter %P increased through time ($p < 0.0001$), and with SRP, under both low and high light incubations, but was differentially affected by variable light intensity (Table 1). Light availability significantly influenced litter P content under high-P concentrations only, where mean %P was approximately0.18% under low light and 0.26% under high light treatments. Litter C content ranged from approximately 43 to 50% throughout the study and varied relatively little during the first 70 days, then increased slightly under low light, low-P enrichments and decreased slightly under high light, mod.- and high-P conditions. Litter N content increased through time and with P enrichment, but was not significantly influenced by light level, and by the end of the study ranged from approximately 1.6 to 2.7%.

The increase in P content resulted in subsequent decreases in C:P across treatments (Fig. 1). Results from the repeated-measures ANOVA showed both a time ($p < 0.0001$) and time*treatment effect ($p < 0.0001$) and differences in litter stoichiometry began on d 29. One-way ANOVAs for each treatment by day showed that the moderate- and high-P treatments in both light levels reached minima by d 36, where C:P (e.g. $C:P_{min}$) remained statistically similar

for the duration of the study. Litter C:P incubated in low-P enrichment varied little through time for both low and high light treatments (approximately 3819 ± 687 and 3740 ± 993, respectively). Within both light levels, $C:P_{min}$ decreased with each level of increasing water P concentration and were as low as 660 ± 237 and 580 ± 387 in the low and high light, high-P treatments, respectively. Light significantly influenced litter C:P only in the moderate-P treatments (1720 ± 536 and 2175 ± 432 for low and high light treatments, respectively). Leaf litter mean N:P ranged from 20 to 90 and was significantly influenced by P enrichment and light availability, following an identical statistical pattern to C:P (Table 1). Mean litter C:N ranged from 30 to 43 and decreased significantly with increasing P concentrations, but was not influenced by light availability.

Algal biomass changed through time ($p < 0.0001$) and there was a significant time*treatment interaction ($p < 0.0001$). Algal biomass was stimulated along the P gradient, but only when coupled with high light availability (Fig. 2). In the high light incubations, chlorophyll *a* peaked at 1.7 and 2.4 μg cm^{-2} in the moderate- and high-P treatments, respectively. But, algal biomass was not stimulated with increased light availability in the low-P treatment.

Respiration rates were affected by time ($p < 0.0001$) and there was a time*treatment interaction ($p < 0.0001$). Respiration rates increased with increasing P concentrations but were not influenced by light availability ($F_{5, 136} = 10.3$, $p < 0.0001$; Fig. 3). Mean respiration rates across P treatments ranged from 0.11 to 0.31 μg O_2 mg $AFDM^{-1}$ h^{-1} and 0.12 to 0.31 μg O_2 mg $AFDM^{-1}$ h^{-1} in low and high light treatments, respectively. There was also a significant time ($p < 0.0001$) and time*treatment ($p < 0.0001$) interaction effect for net metabolic rates. Rates increased significantly along the P concentration gradient in the high light treatments but did not differ among P concentrations in the low light treatments ($F_{5, 138} = 16.4$, $p < 0.0001$; Fig. 4). Net

photosynthesis occurred in high light, moderate- and high-P treatments, while net respiration occurred across all P concentrations in low light treatments (Fig. 4).

Carbon:P ratios were significantly correlated with net O_2 flux when P treatments within each light level were considered collectively (Fig. 5). Low light treatments followed a negative linear pattern ($F_{2, 21} = 25.6$, $p < 0.0001$), where litter C:P decreased as net O_2 flux became more negative and as P concentrations increased. High light treatments followed an exponential decay model ($F_{2, 21} = 18.6$, $p < 0.01$), where litter C:P decreased as net O_2 flux became more positive and as P concentrations increased.

Phosphorus uptake rates increased with time ($p < 0.0001$), but were not affected by treatment ($p > 0.05$). Mean uptake rates among treatments ranged from 5.1-9.2 ng P cm^{-2} h^{-1} and were highly variable across replicates (Table 2). The proportion of P released to total P uptake was also highly variable, generally very low (< 10%) and did not differ among treatments (Table 2). Carbon assimilation rates and NPP rates were significantly correlated with P uptake rates for low and high light treatments, respectively (Fig. 6). High light treatments were more strongly correlated and had higher slopes compared to low light treatments. In the high light treatments, the slopes were unequal and increased with each increasing P concentration ($F_{5, 71} = 27.2$, $p < 0.0001$). But, slopes in the low light treatments were not different ($F_{5,75} = 0.17$, $p > 0.1$), demonstrating that there were no significant differences in the C_{assim} – P uptake relationships as a function of treatment. But, after adjusting for P uptake, there were differences in C_{assim} among treatments ($F_{3, 75} = 23.0$, $p < 0.0001$). We examined these relationships to estimate the C:P of assimilated nutrients ($C:P_{assim}$) by using linear regression and assuming the slope to equal $C:P_{assim}$ for each treatment.

Stream nutrient concentrations varied throughout the study period. Mean SRP concentration was 10.6 µg/L (range 2-26), while values for ammonium and nitrate were 30 µg/L (range 12-70) and 497 µg/L (range 213-1090), respectively. Mean DOC concentration was 500 µg/L (range 387-818). Mean light levels were 14 µmol photons m^{-2} s^{-1} (range 10-17) and 475 µmol photons m^{-2} s^{-1} (range 400-595) for low and high light incubations, respectively.

D. Discussion

The objective of this study was to quantify the effect of P and light availability on leaf litter stoichiomety. Further, we wanted to determine the mechanisms that may be involved, including the presence of algae, altered metabolic regimes, and P uptake and release rates. Our results support the general hypothesis that litter C:P would be differentially influenced by water column P concentrations and light availability. Litter C:P decreased with increasing levels of P enrichment, and there was a significant influence of light on litter quality. Additionally, factors that influence algal activity (i.e. light and nutrients) were shown to affect leaf biofilm metabolism, ultimately driving changes in litter C:P.

1. *Litter stoichiometry*

Anthropogenic activities are increasingly affecting stream ecosystems by means of increased nutrient loading from agriculture and urbanization and altered light regimes from riparian canopy loss. Microbial-mediated litter conditioning is generally considered a heterotrophic process, but our results indicate that litter quality can be influenced not only by changes in nutrient concentrations, but also that light availability can further affect these changes. Litter %P increased with increasing P enrichment in both light levels and was highest

when high P enrichment was coupled with high light availability. Other studies have shown an increase in litter P content with increasing nutrient concentrations (Elwood et al. 1981, Cross et al. 2003), presumably as a result of enhanced microbial activity. Litter %P was generally within the range of values reported by other studies (approximately 0.05-0.22%; Evans-White et al. 2005, Small and Pringle 2010), with the exception of high light, high-P treatments (max., 0.62%). This could be a result of high algal biomass accrual and possibly associated luxury P uptake by algae (Sterner and Elser 2002), although this could not be supported with our P uptake data.

Increasing P enrichment led to decreasing litter C:P, but this response was different across light levels. Litter C:P decreased to a minimum ($C:P_{min}$) by day 36, where elemental changes leveled off and there were no further changes for the duration of the study. This occurred in all treatments except for the low-P, low and high light treatments, in which litter C:P followed no changing trend through time. Litter C:P in our study was within the range of values reported by others (approximately 450 to 7000; Cross et al. 2003, Evans-White et al. 2005, Small and Pringle 2010). Carbon:P_{min} ratios decreased with each increasing level of P enrichment under both light conditions. There was a significant light effect only with mod.-P enrichment, where high light resulted in higher $C:P_{min}$. Percent P and C loss as respiration did not differ among these two treatments, which suggests that algal biomass (e.g. C assimilation from photosynthesis) may have driven this response under high light conditions. Algae were likely P-limited in the mod.-P treatment (molar N:P 56), which, under high light conditions, can result in nutrient poor algae (Sterner and Elser 2002, Fanta et al. 2010). It is important to note that while $C:P_{min}$ was similar in high-P enrichments under both light incubations, litter quality was likely

very different, given high algal biomass (e.g. labile OC) associated with leaves incubated under high light (Franken et al. 2005).

Leaves were incubated in microcosms that lacked flow through to constantly replenish nutrient supply. Therefore, one potential limitation of our experimental design was that the experimental units could have experienced an extreme drawdown of ambient nutrients that may have resulted in more severe nutrient limitation, especially in the low- and mod.-P treatments. We tested this hypothesis in association with another study. Results demonstrated that while ambient concentrations of SRP quickly decreased to below detection between stream water replenishments, mean water column TP concentrations were approximately 45 and 65 µg/L in the low- and mod.-P treatments, respectively. This suggests that P turnover from the water column was probably an important P source to litter, similar to conditions often observed in streams (Dodds 2003).

2. *Algal biomass and microbial metabolism*

Light and nutrients commonly limit primary production in streams. Our prediction that algal biomass would increase with increasing light and nutrients was only partially supported. Algal biomass was minimal and not stimulated by P enrichment under low light conditions similar to a stream with dense riparian vegetation (approximately 15 µmol photons m^{-2} s^{-1}), suggesting that light was the primary limiting factor. Algae were only stimulated under high light conditions when coupled with P enrichment. This result could be an artifact of heterotrophic microbes outcompeting algae at low nutrient concentrations (Currie and Kalff 1994). Elevated algal biomass only occurred under high light when stream water P was ≥ 50 µg/L. Under high light and high nutrient concentrations, algal growth on organic matter was

similar to that reported by other studies (chlorophyll *a* concentrations, 0.06 – 4.2 µg /cm^2; Sabater et al. 1998, Albariño et al. 2008).

Microorganisms can greatly impact C and energy flow through lotic ecosystems due to their metabolic activities. Respiration rates ranged from approximately 0.02-0.6 µg O_2 mg $AFDM^{-1}$ h^{-1}, generally within the range of values reported in other studies (approximately 0.1 – 0.45 µg O_2 mg $AFDM^{-1}$ h^{-1}; Gulis and Suberkropp 2003, Stelzer et al. 2003). Phosphorus enrichment stimulated respiration rates in both light levels, but there was no interactive effect of light. Respiration rates can be a good indicator of microbial activity, and by this measure, our results suggest that heterotrophic microbial activity may not have been enhanced by the presence of algae. But, it cannot be determined with certainty whether algae influenced heterotrophic activity in our study. Considering respiration rates alone may be an insufficient method to detect changes in heterotrophic activity, and measurements of bacterial and fungal biomass may provide a more realistic account of potential differences. Measures of fungal and bacterial biomass have been reported in studies demonstrating algal stimulation of heterotrophic activities and decomposition (Neely 1994, Lagrue et al. 2011). However, Albariño et al. (2008) reported no change in bacterial biomass and decreases in fungal biomass under high light conditions. Light levels were very high in their study (maximum PAR 2000 µE m^{-2} s^{-1}) and they suggested that this resulted in inhibitory effects of UV radiation on fungi (Albariño et al. 2008).

Algal presence on organic substrate has the capacity to affect decomposition through the priming effect (PE). Priming occurs when the degradation of recalcitrant OM increases in the presence of labile OC (Guenet et al. 2010). Allochthonous food resources such as leaves are considered to have inherently poor quality and have a range of recalcitrant C compounds that may cause C-limitation of microbes (Ardón and Pringle 2007). In high light treatments where

algal growth was moderate, labile C exudates from algae could have stimulated leaf breakdown and thus fungal and bacterial activities. However, our respiration results did not provide evidence that PE occurred. Heterotrophic microbes are capable of utilizing DOC from the water column and in streams with high concentrations of DOC, microbes rely less on leaf material for their C demand (Baldy et al. 2007) and might not be affected by further increases in available DOC (e.g. from algal exudates). However, this is likely not the case in our study given relatively low DOC concentrations (500 µg/L). On a spectrum of C degradability, maple leaves tend to be more labile, providing more available C to decomposers (Melillo et al. 1982). Carbon may not have been limiting to microbes and thus additions of labile C from algae would not have influenced heterotrophic activity. However, while we lack data that provides evidence of PE, due to anecdotal observations of apparently greater decomposition under high light, and especially high-P, treatments, we think that there was a positive effect of algae on heterotrophic activity. Future studies should include measures of microbial biomass, enzyme activities, and decomposition rates, and should consider a range of leaf species of variable degradability to better address the impact that priming might have on litter conditioning.

Light and nutrients significantly influenced net metabolic rates. All low light treatments exhibited net respiration throughout the study, as expected given minimal algal biomass associated with these treatments. Net metabolism remained near zero under high light, low-P incubations, but when nutrients were added along with high light availability, metabolism shifted to net photosynthesis. This is similar to results reported by Sabater et al. (1998). In their study, net metabolism associated with wood biofilms were negative (respiration dominated) under light levels of approximately 350 µmol photons m^{-2} s^{-1}, but net metabolism became positive (photosynthesis dominated) after N addition (Sabater et al. 1998). Algal activity may have been

suppressed due to competition with heterotrophic microbes under low nutrient concentrations, and this limitation may have been relieved with nutrient enrichment when light was not limiting. Litter C:P was strongly correlated with net metabolism, demonstrating that either heterotrophic and autotrophic microbial colonization drove changes in C:P and that these changes occurred at different rates when leaves were incubated in low (linear rate of change) versus high light (exponential decay rate of change) conditions. Nutrient enrichment drove metabolism to 2 different extremes with shaded conditions resulting in large amounts of O_2 being consumed and high light conditions resulting in large amounts of O_2 being produced. Net metabolism converged under low-P treatments where net O_2 flux was slightly negative, and thus net heterotrophic. The interaction between increasing light and nutrients might result in large ecosystem shifts in metabolic regimes and C cycling.

3. *Phosphorus cycling and carbon assimilation rates*

Our measured P uptake rates increased through time and ranged from approximately 0.6-20 ng P cm^{-2} h^{-1}, near the range of values reported in other studies (0.7-10 ng P cm^{-2} h^{-1}; Newbold et al. 1983, Mulholland et al. 1984, Mulholland et al. 1985, Webster et al. 2009). We expected to measure net P release during later stages of decomposition (Webster et al. 2009, Cheever et al. 2012), but instead we measured continued net P uptake and were unable to reliably detect net P mineralization. Cheever et al. (2012) reported net P mineralization with more frequency than net uptake, but described that these fluxes might have been an artifact of the precision of their analytical methods. The maximum values of %P released (e.g. proportion of P released to total P taken up) were very low (approximately 5-20%) and highly variable. Furthermore, due to our experimental design, we were unable to filter these samples which likely

led to an overestimation of release from interference by radioactivity in particulate OM. These results suggest strong internal recycling of nutrients within leaf biofilms and little net release of dissolved P back to the water column.

We plotted C assimilation and P uptake rates to estimate the slopes of these relationships which should equal the ratio of assimilated C:P. However, these data did not seem to accurately portray reality. Assimilation of C was different among P treatments for both low and high light incubations, but P uptake rates were not different among treatments. Carbon assimilation rates for low light treatments were calculated using a factor of 0.6 for microbial growth efficiency which likely grossly over- and underestimated actual assimilation rates that should vary among treatment conditions. We used NPP as a proxy for C assimilation in high light treatments, which may not have been the most appropriate method. The use of labeled C to trace both autotrophic and heterotrophic C assimilation could yield promising results. Additionally, we feel that our P uptake data may not have accurately captured differences among treatments due to methodological constraints. But, using a modified approach, we think that this type of analysis could elucidate treatment differences in C:P assimilation rates that should correspond to changing litter quality.

4. Conclusion

Our results demonstrate that moderate P enrichment (50 µg SRP/L) can significantly alter microbial-mediated litter stoichiometry and quality, and that these changes are exacerbated with increasing P enrichment (500 µg SRP/L). Greater light availability, simulating riparian canopy loss, interacted with P enrichment to differentially effect litter processing and quality. Furthermore, we observed shifts in metabolic regimes from net heterotrophic (respiration

dominated) to net autotrophic (photosynthesis dominated) depending on whether leaves were incubated under shaded or open canopy conditions. Intact riparian vegetation is important to maintain the integrity of forested stream ecosystems, and if not managed appropriately, alterations to litter processing and C cycling should be expected. Our results can add to the growing scientific evidence available to guide environmental decisions and nutrient criteria for aquatic ecosystems.

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F. Acknowledgements

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G. Tables

Table 1. Results from ANOVAs comparing effect of phosphorus concentration and light availability on algal biomass ($F_{5, 138} = 25.12$), litter P content ($F_{5, 138} = 38.14$), litter N:P ratio ($F_{5, 138} = 90.7$), and minimum litter C:P ($C:P_{min}$; $F_{5,12} = 115.6$, $p < 0.0001$). Values are means of data beginning on day 29 (except for $C:P_{min}$ which are means beginning on day 36, when C:P remained similar for the duration of the study), with the range in parentheses. Treatments with the same letters within a property were not statistically different. An asterisk (*) denotes treatments that showed no changing trend through time for $C:P_{min}$.

Treatment		Algal biomass ($\mu g/cm^2$)		%P		N:P		$C:P_{min}$	
Low light	Low-P*	0.02 (0.01-0.05)	A	0.03 (0.03-0.04)	A	90 (58-117)	A	3820 (3026-4465)	A
	Mod.-P	0.04 (0.01-0.15)	A	0.07 (0.04-0.12)	A	50 (44-57)	C	1720 (1048-2330)	C
	High-P	0.04 (0.01-0.23)	A	0.18 (0.05-0.25)	B	26 (21-45)	D	660 (496-1127)	D
High light	Low-P*	0.10 (0.01-0.23)	A	0.03 (0.02-0.05)	A	95 (74-146)	A	3740 (2574-5167)	A
	Mod.-P	0.50 (0.07-1.74)	B	0.05 (0.03-0.06)	A	69 (53-83)	B	2175 (1935-2534)	B
	High-P	1.06 (0.21-2.43)	C	0.26 (0.07-0.57)	C	20 (10-37)	D	580 (195-1359)	D

Table 2. Mean phosphorus uptake rates and proportion of maximum P released to total litter P uptake (as a percentage) across all treatments. Ranges are in parentheses.

Treatment		P uptake rate (ng P cm-2 h-1)	%P released
Low light	Low-P	6.7 (1.6-13.9)	13.6 (8.3-29.8)
	Mod.-P	6.9 (1.4-15.4)	7.8 (2.6-16.3)
	High-P	5.1 (1.2-15.3)	11.3 (4.6-17.1)
High light	Low-P	5.1 (1.9-7.7)	14.4 (2.9-27.3)
	Mod.-P	9.2 (1.9-18.8)	6.5 (1.7-11.6)
	High-P	6.4 (1.4-18.3)	8.4 (1.6-18.5)

H. Figure legends

Figure 1. Scatter plot of carbon:phosphorus (C:P) molar ratio of (a) low light and (b) high light treatments through time. Triangles represent low-P, circles represent mod.-P, and squares represent the high-P treatment. Error bars represent ± 1 SD. Letters represent statistical differences among treatments.

Figure 2. Scatter plot of chlorophyll *a* through time. Black and white symbols represent low and high light treatments, respectively. Triangles represent low-P, circles represent mod.-P, and squares represent the high-P treatment. Error bars represent ± 1 SE. Letters represent statistical differences among treatments.

Figure 3. Scatter plot of respiration rates through time for (a) low light and (b) high light treatments. Triangles represent low-P, circles represent mod.-P, and squares represent the high-P treatment. Error bars represent ± 1 SE. Letters represent statistical differences among treatments.

Figure 4. Scatter plot of net O_2 flux through time. Black and white symbols represent low and high light treatments, respectively. Triangles represent low-P, circles represent mod.-P, and squares represent the high-P treatment. Error bars represent ± 1 SE. The horizontal line indicates the shift from net photosynthesis (above this line) and net respiration (below this line). Letters represent statistical differences among treatments.

Figure 5. Scatter plot of the relationship between litter C:P and net O_2 flux. Data points are means for each treatment beginning on day 29. Regression lines are fit for each light level, with symbols indicating P treatments within each light level. Black and white symbols represent low and high light treatments, respectively. Triangles represent low-P, circles represent mod.-P, and squares represent the high-P treatment. The vertical line indicates the shift from net heterotrophic (left of the line) to net autotrophic (right of the line). Correlations were significant for both low (linear, equation: $y = 8659x + 4170$, $r^2 = 0.52$, $p < 0.0001$) and high light (exponential decay, equation: $y = 2885^{-1.53x}$, $r^2 = 0.43$, $p < 0.01$) treatments.

Figure 6. Scatter plot of the relationship between estimated carbon (C) assimilation rates (for low light treatments) or net primary production (for high light treatments, proxy for C assimilation) and P uptake rates. Regression lines are fit for each treatment. Correlations are significant for all treatments and statistical values are: low light: low-P ($r^2 = 0.50$, $p < 0.0001$, slope = 12.2), mod.-P ($r^2 = 0.28$, $p < 0.01$, slope = 10.2), high-P ($r^2 = 0.14$, $p < 0.05$, slope = 13.5); high light: low-P ($r^2 = 0.34$, $p < 0.001$, slope = 19.5), mod.-P ($r^2 = 0.70$, $p < 0.0001$, slope = 54.9), high-P ($r^2 = 0.85$, $p < 0.0001$, slope = 120.1). Circled data points are apparent outliers and were removed from the analysis.

I. Figures

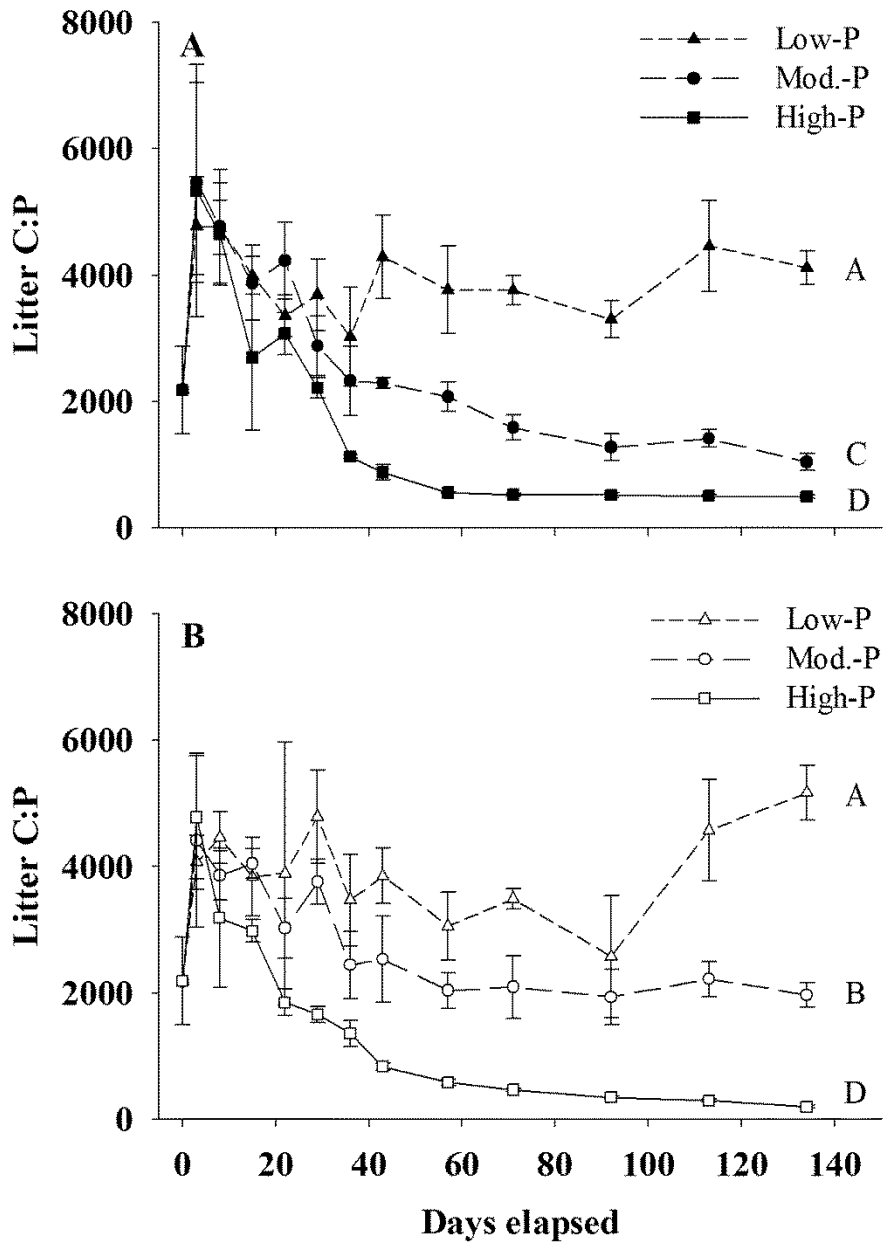

Figure 1

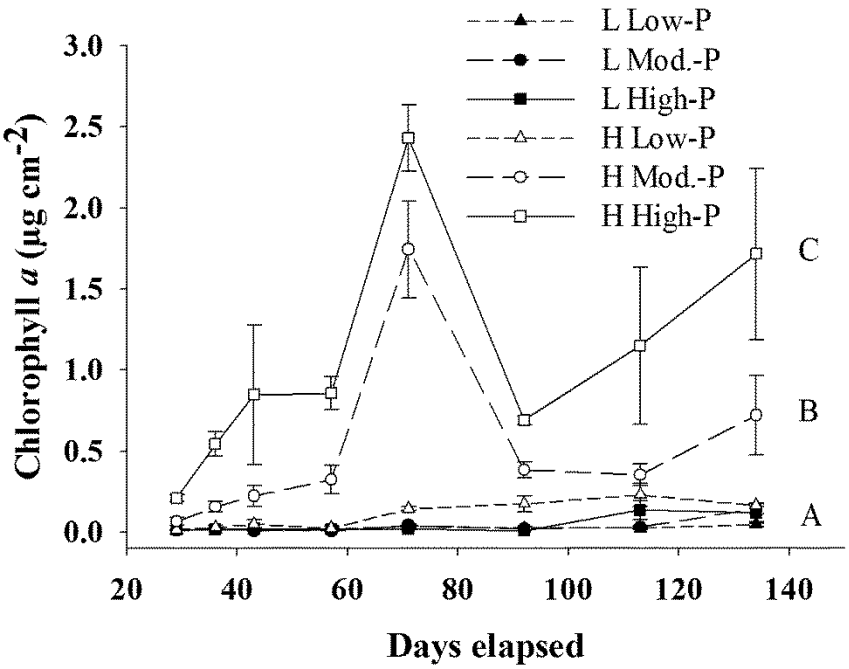
L Low-P
L Mod.-P
L High-P
H Low-P
H Mod.-P
H High-P
Chlorophyll a (µg cm-2)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
20
40
60
80
100
120
140
Days elapsed
C
B
A

Figure 2

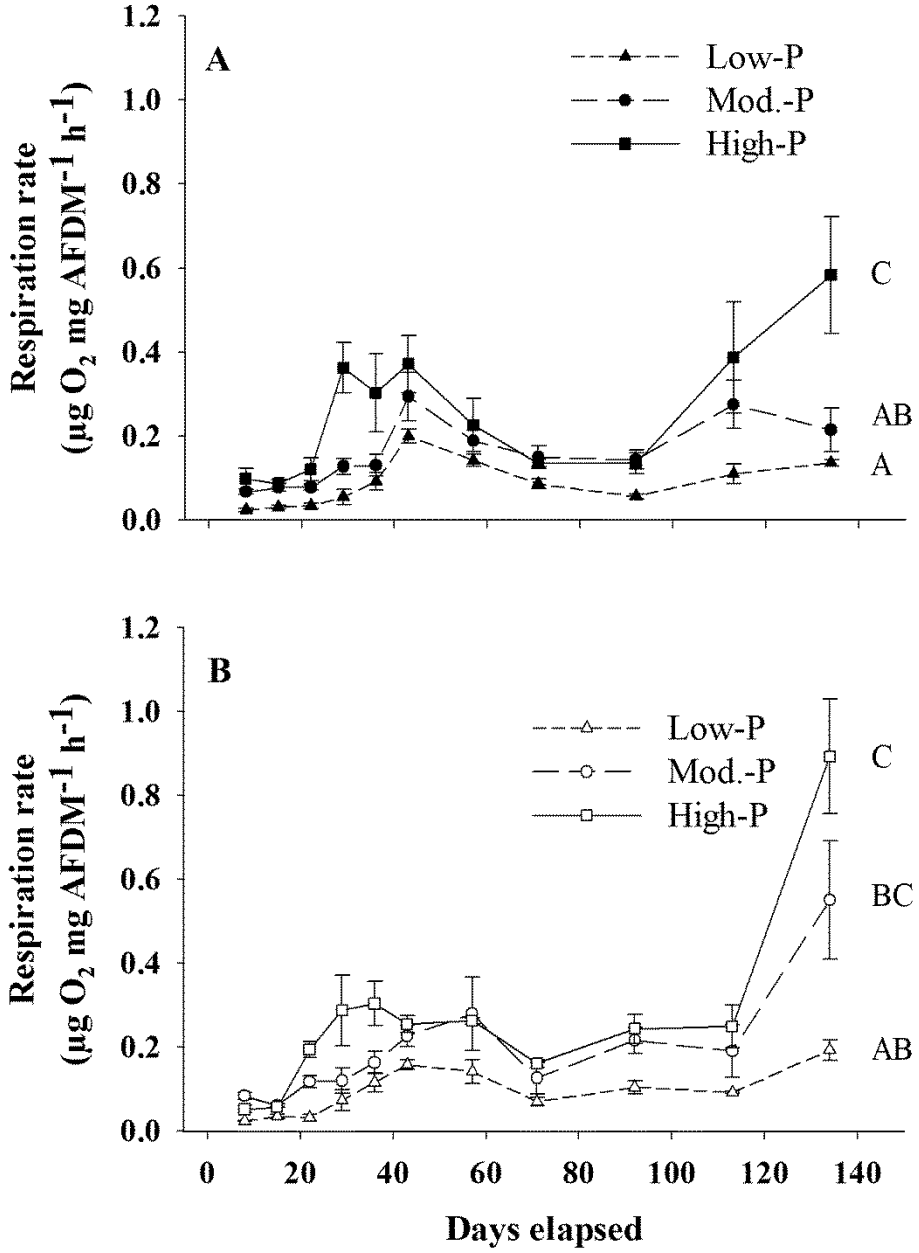
A
Low-P
Mod.-P
High-P
Respiration rate
(μg O2 mg AFDM-1 h-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C
AB
A
B
Low-P
Mod.-P
High-P
C
BC
AB
0
20
40
60
80
100
120
140
Days elapsed

Figure 3

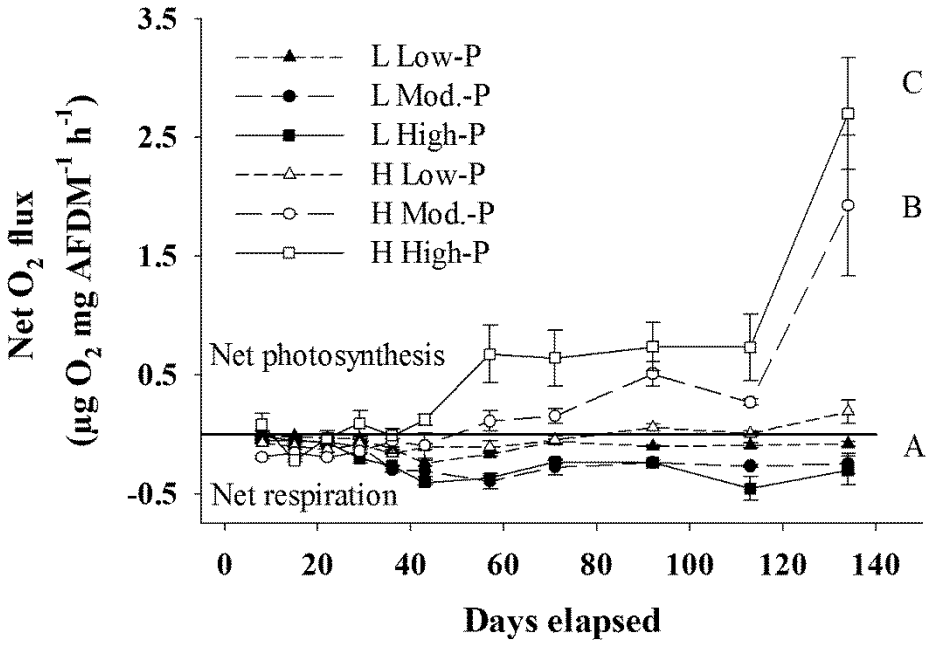
3.5
2.5
1.5
0.5
-0.5
Net O_2 flux
(μg O_2 mg AFDM^{-1} h^{-1})
L Low-P
L Mod.-P
L High-P
H Low-P
H Mod.-P
H High-P
Net photosynthesis
Net respiration
C
B
A
0
20
40
60
80
100
120
140
Days elapsed

Figure 4

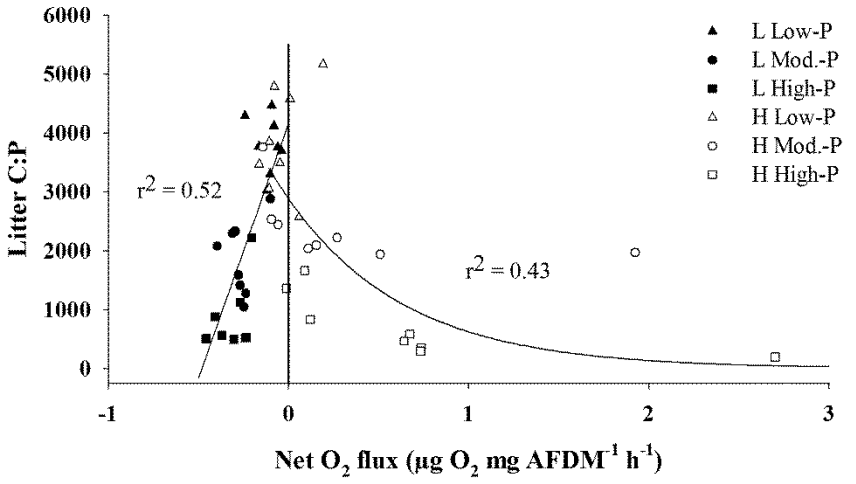
6000
5000
4000
3000
2000
1000
0
Litter C:P
-1
0
1
2
3
Net O2 flux (µg O2 mg AFDM-1 h-1)
r2 = 0.52
r2 = 0.43
L Low-P
L Mod.-P
L High-P
H Low-P
H Mod.-P
H High-P

Figure 5

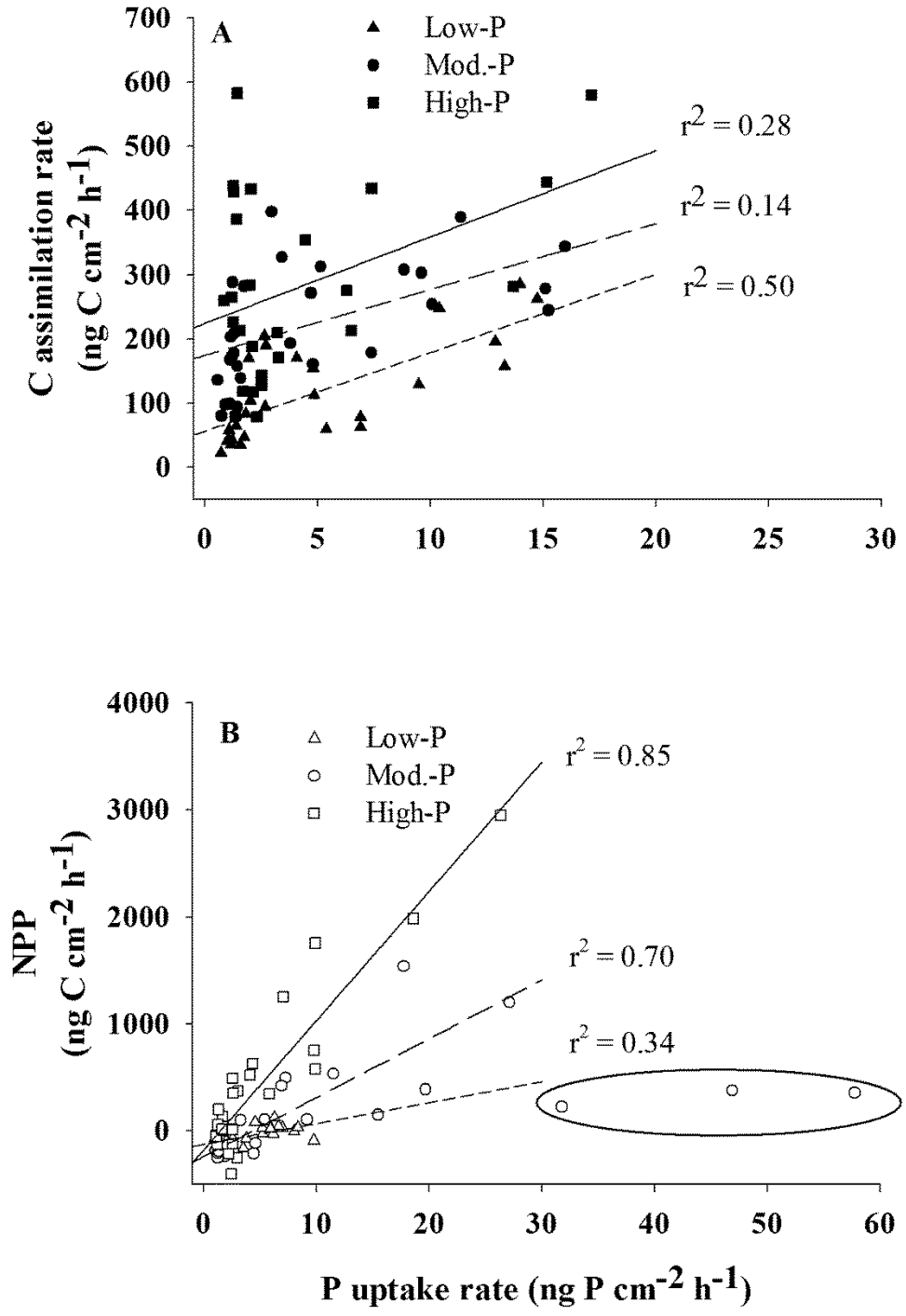
A
Low-P
Mod.-P
High-P
$r^2 = 0.28$
$r^2 = 0.14$
$r^2 = 0.50$
C assimilation rate (ng C cm^{-2} h^{-1})
0
100
200
300
400
500
600
700
0
5
10
15
20
25
30
B
Low-P
Mod.-P
High-P
$r^2 = 0.85$
$r^2 = 0.70$
$r^2 = 0.34$
NPP (ng C cm^{-2} h^{-1})
0
1000
2000
3000
4000
0
10
20
30
40
50
60
P uptake rate (ng P cm^{-2} h^{-1})

Figure 6

J. Appendix

I hereby state that Erin E. Scott was the lead author on chapter 3 of this thesis titled “Phosphorus concentration and light availability differentially affect microbial-mediated leaf litter conditioning” and that she completed at least 51% of this work.

J. Thad Scott
Thesis Director

IV. CONCLUSION

The primary objective of these studies was to quantify the response of litter C:P to multiple environmental factors including phosphorus (P) enrichment, leaf species, and light availability (to simulate shaded and open canopy systems). Numerous studies have demonstrated the response of bacterial and fungal activities (e.g. biomass accrual, respiration rates, and decomposition rates) to alterations in environmental factors, but few have explicitly quantified the change in litter stoichiometry, and thus quality. This study demonstrated that litter C:P decreased through time to saturation and that the magnitude of this change was differentially influenced by leaf species and light availability. Also, increased light intensity resulted in greater algal biomass and changes in metabolic activities.

The results of this study show that initial leaf carbon (C) compositional properties influenced the response of litter C:P to increasing P concentration. Specifically, enrichment to more labile maple leaves resulted in lower litter saturation C:P ($C:P_{sat}$) compared to oak leaves. This was attributed to the recalcitrant nature of oak leaves and the possibility that microorganisms may have exhausted the available C supply quickly, and may have been C-limited for the duration of the study. In both studies, when no P was added, litter C:P showed no trend through time and values were more variable. When the response of litter C:P to P enrichment and light availability was tested, $C:P_{sat}$ decreased with increasing P availability, but light significantly influenced litter C:P only under moderate-P concentrations. With moderate-P enrichment, litter P content was similar in both light levels. But, under high light incubations, litter C:P was greater than under low light incubations, likely a result of moderate algal biomass accrual and C assimilation via photosynthesis. Also, high light intensity resulted in metabolic shifts from net respiration (e.g. heterotroph dominated) to net photosynthesis (e.g. autotroph

dominated). These studies indicate that perturbations to nutrient and light regimes can result in significant alterations to detrital quality and biogeochemical cycles of C and P.

Maple leaves were used in both studies and the response to nutrient enrichment was similar, but there was a greater decrease in C:P for all P concentrations in the first experiment compared to the second. However, a couple of environmental factors important to microbial activity varied between the 2 studies. The first was conducted at ambient room temperature (approximately 20°C) with no added N above stream background concentrations, while the second was conducted at 10°C with 1 mg DIN/L added to all experimental units. The initial range in maple C:P was 1000 to 6000 and 1000 to 3000 for the first and second experiment, respectively. This suggests that microbial activity may have been more temperature limited rather than N-limited.

The results of these studies demonstrate the importance of proper nutrient management and diverse and continuous riparian vegetation to maintain the integrity of forested stream ecosystems. Modifications of nutrient and riparian regimes can affect litter conditioning, C fluxes, and resource quality. Altering these basic ecosystem processes will likely influence consumers, resulting in shifts in community composition and biodiversity losses.