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Effects of Water Column P-availability and Litter Microbial Mediated Processes and Stoichiometry in Aquatic Systems

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Effects of Water Column P-availability and Litter Microbial Mediated Processes and
Stoichiometry in Aquatic Systems

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by

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ABSTRACT

Anthropogenic nutrient enrichment of aquatic ecosystems is prevalent and can have detrimental effects on biological condition. Many of these effects have been attributed directly or indirectly to changes in autotrophic processes. However, in detrital-based streams changes in course detrital stoichiometry and decomposition have been related to changes community structure and production at higher trophic levels highlighting the importance of considering the heterotrophic microbial responses to enrichment. Leaf litter stoichiometry, which is determined in part by the tree species source and the microbial communities that comprise the associated biofilm, is likely related to decomposition rates, but few studies examine these variables in combination across a nutrient concentration gradient. Dissolved inorganic phosphorus (DIP) (0, 10, 25, 50, 75, 100, 250, 500, 1000, 2000 µg/L DIP) and litter type (sugar maple and post oak) were manipulated in a 210 day laboratory microcosm study to determine their effect on litter C:P and decomposition rates and to examine their effects on short duration measures of respiration and alkaline phosphatase activity (APA) taken on several dates spanning the decomposition process. I found that C:P declined and decomposition rates increased in a saturating relationship along the DIP gradient indicating P-limitation of decomposition at low DIP concentrations. Saturation values for maple and oak C:P and decomposition rates were 500 and 830 and 0.0085 $\pm$ 0.0005 and 0.0029 $\pm$ 0.0002, respectively. Litter C:P saturation DIP concentrations saturated at approximately 80 µg/L for both litter types, while decomposition rates saturated at less than 25 µg/L for both litter types. APA and respiration rates were not correlated, but positive correlations were found between C:P and APA on several days (range in r = 0.568-0.670, p<0.008). Positive correlations between respiration rates and decomposition across several days but explained little variation in decomposition rate (r<0.5) Maple tended to have greater APA and respiration relative to oak litter, though not always statistically. These results demonstrate that litter type and

DIP concentration are both important factors affecting litter stoichiometry and quantity in detrital systems, but do not support the use of APA and respiration as instantaneous measures of limitation of decomposition in streams. I provide insight into the complexity of the dynamics of nutrient enrichment in stream, educating potential impacts of nutrient loading on aquatic ecosystems.

ACKNOWLEDGEMENTS

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I.   INTRODUCTION

Anthropogenic nutrient enrichment of aquatic ecosystems is a pervasive occurrence that continues to expand with the growing human population and the associated conversion of land for agricultural and urbanization. Negative effects of excess nutrient loading are generally attributed directly or indirectly to autotrophic processes including increased toxic algal blooms, decreased dissolved O_2 concentrations, increased turbidity, and declines in species diversity (Smith 2003, Dodds et al. 2009), while effects on detrital based systems have been less widely addressed (Evans-White et al. 2013).

Low-order forested streams rely on autochthonus inputs, such as leaf litter, as a primary source of carbon (C) and energy for overall production (Fisher and Likens 1973, Vannote et al 1980). Such streams are often characterized by low water column nutrient concentrations (Elwood et al. 1981, Baldy et al. 2007) thereby leaving biota potentially nutrient limited. Therefore, increasing nutrients can lead to changes in biotic processes, potentially affecting ecosystem functioning. Recent studies have shown that nutrient enrichment increases the nutrient content of detrital resources (Cross et al. 2003, Scott et al 2013) and increases the microbial-mediated decomposition of detritus (Gulis and Suberkropp 2003, Güsewell and Gessner 2009, Woodward et al. 2012). Such changes in detrital quality and quantity have been seen in conjunction with shifts and declines in macroinvertebrate community structure and biodiversity, respectively (Singer and Battin 2007, Evans-White 2009). Dose-response studies linking detrital quality, such as stoichiometry, and quantity responses are lacking and would aid wadeable stream water quality management.

A dietary imbalance of nutrient elements in the detritus-detritivore interaction may explain some of the effects of nutrient enrichment on detritivorous functional feeding group taxa

(Evans-White et al. 2009, Danger et al. 2013, Halvorson et al. in press). Resorption of nutrients

prior to senescence results in leaf litter that is very low in nutrient content when it enters the

stream (Aerts 1996; Vergutz et al. 2012) when compared to consumer demand resulting in a

large consumer-resource imbalance which can constrain consumer growth (Sterner and Elser

2002, Cross et al. 2003; Halvorson et al. in press). Many detritivores have adapted to rely on this

detrital resources and it has been found that increasing detrital quality can be associated with

negative impacts on detritivorous macroinvertebrates, including decreases in species richness

(Evans-White et al. 2009), potentially due to the lack of some species to cope with increasing

nutrient content of their food (Boersma and Elser 2006, Halvorson et al. in press). While these

findings suggest a mechanistic link between nutrient enrichment and consumer responses, it is

often difficult to assess the effect of enrichment-driven changes in detrital stoichiometry on

macroinvertebrate detritivores because changes in quality often coincide with changes in detrital

quantity.

Stimulation of microbial processes in response to nutrient enrichment have shown

increased microbial respiration and decomposition rates (Elwood et al. 1981, Gulis and

Suberkropp 2003, Güsewell and Gessner 2009, Gulis 2006,Suberkropp 2010, Woodward et al.

2012). Suberkropp et al. (2010) showed that when compared to a reference stream, nutrient

enrichment resulted in an increase in fungal and bacterial productivity and microbial respiration

rates. These responses were associated with much faster decomposition rates, decreased leaf

litter standing stock, and limited food web production. Increases in leaf litter nutrient content

(Cross et al. 2003) via immobilization of nutrients from the water column by microbes

(Suberkropp 1998; Gulis and Suberkropp 2003) and microbial responses, such as increased

decomposition rate, to increased nutrients (Elwood 1981, Suberkropp et al. 2010) may play a

dynamic role in the effects of nutrient enrichment on aquatic ecosystems. Quantitative relationships between nutrient concentration, litter stoichiometry, and decomposition rates are needed to determine at what levels quality and quantity effects on higher trophic levels may be important.

Quantifying decomposition is a labor intensive process and may not be feasible in a management setting. Litter stoichiometry, respiration rates, and alkaline phosphatase activity (APA) measures are less labor intensive and may adequately identify nutrient limitation of decomposition. The elemental composition or stoichiometry of algae has been frequently used to assess nutrient limitation of algal growth (Sterner and Elser 2002). It is possible that it may provide a useful indicator of limitation status of decomposition if background litter species effects on detrital stoichiometry are also determined. Increasing microbial biomass (Rosemond et al. 2002, Benstead et al. 2005) and respiration rates (Greenwood et al. 2007, Suberkropp et al. 2010) have been associated with enrichment and can modify detrital stoichiometry and decomposition. Further, studies have shown that microbial phosphatase activity responds to nutrient enrichment with increased activity under P-limited conditions (Healey and Hendzel 1980; Chrost and Overbeck 1987; Hill et al. 2006). All of these measurements are far less time-consuming than are decomposition measures. Therefore, understanding patterns in stoichiometry, APA, and respiration, along with decomposition rates, across a wide P gradient may provide more instant and, therefore, widespread assessments of microbial nutrient limitation, better educating the development of nutrient criteria and management strategies.

I conducted a 210 day microcosm study to1) determine the response of litter C:P and microbially-mediated decomposition rates, 2) examine the effects of time and P-enrichment on respiration rates and alkaline phosphatase activity (APA), and 3) determine whether these short

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term measures were related to litter stoichiometry and decomposition rates for two leaf types (sugar maple and post oak) across a (dissolved inorganic phosphorus) DIP gradient (0, 10, 25, 50, 75, 100, 250, 500, 1000, 2000 µg/L DIP). I hypothesized that 1) the minimum litter C:P observed over time would decrease with increasing P enrichment and decomposition rates would increase with increasing P enrichment in a saturating manner across the P gradient, 2) maple litter would reach minimum C:P and maximum decomposition rate at a lower P concentration than on the more recalcitrant oak leaves due to differences in C availability between the litter species, 3) APA would be higher at lower P concentrations and would decrease for all P treatments over time, while respiration rates would increase across the P-gradient and through time, 4) APA and respiration rates would be greater for maple leaves across the P gradient and through time, and 5) APA and C:P ratios would be positively related and APA and respiration rates inversely related across the P gradient, while respiration rates would be positively related to decomposition rates.

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II. THE EFFECTS OF PHOSPHORUS AVAILABILITY AND LITTER SPECIES ON
MICROBIAL PROCESSES AND LEAF LITTER STOICHIOMETRY

A. Abstract

In forested streams that rely on allochthonous detrital material as a primary source of energy, anthropogenic nutrient enrichment can result in changes in detrital food quality and quantity. Relationships between litter stoichiometry and microbial processes such as decomposition are rarely examined but are needed to enhance our understanding of nutrient enrichment effects on higher trophic levels. A 210 day microcosm study was performed to determine the response of litter C:P and microbially-mediated decomposition rates for two leaf types (sugar maple and post oak) across a (dissolved inorganic phosphorus) DIP gradient (0, 10, 25, 50, 75, 100, 250, 500, 1000, 2000 µg/L DIP). I found that C:P declined in a saturating relationship through time for all treatments except 0 and 10 µg/L DIP. When plotted along the DIP gradient, C:P values saturated (500 and 830 for maple and oak, respectively) at DIP concentrations of less than 25 µg/L for both litter types. Decomposition rates also increased in a saturating manner across the DIP gradient with breakdown coefficient values of 0.00085 ± 0.005 and 0.00029 ± 0.002 for maple and oak, respectively. Both maple and oak breakdown coefficients (k d^{-1}) saturated at approximately 80 µg/L. ANCOVA results showed that maple litter was significantly higher for both response variables after accounting for DIP effects (F=534.013, p<0.001 and F=13.743, p<0.001) and for maple and oak. These results demonstrate that litter type and DIP concentration are both important factors affecting litter quality and quantity in detrital system. Understanding these relationships and differences in the responses of these variables to enrichment is important in determining potential impacts of nutrient loading on aquatic ecosystems.

B.   Introduction

Anthropogenic nutrient enrichment of aquatic ecosystems is a growing global phenomenon responsible for a number of deleterious effects in streams. Such effects are generally attributed directly or indirectly to autotrophic processes including increased toxic algal blooms, decreased dissolved O_2 concentrations, increased turbidity, and declines in species diversity (Dodds et al. 2009). Dose-response relationships between nutrient concentrations and benthic or planktonic algal biomass have been developed (Dodds et al. 2002) and often serve as the bases for stream nutrient criteria development in the U.S. (Evans-White et al. 2013).

Recent studies, however, have shown strong effects of nutrient enrichment on heterotrophic processes such as increased nutrient content of detrital resources (Cross et al. 2003), increased microbial decomposition of detritus, (Gulis and Suberkropp 2003, Güsewell and Gessner 2009, Woodward et al. 2012), and shifts and declines in macroinvertebrate community structure and biodiversity, respectively (Singer and Battin 2007, Evans-White 2009). Therefore, nutrient enrichment can alter detrital-based food webs and correlate with declines in detritivore biodiversity. However, mechanisms linking stream nutrient enrichment, detrital resource dynamics, and macroinvertebrate compositional shifts and diversity losses are still being identified and assessed.

Bottom-up nutrient enrichment effects on macro-faunal detritivores (>1 mm) may be mediated by changes in detrital quality and quantity (Danger et al. 2013, Fuller unpublished, Halvorson in press). Allochthonous organic material in the form of senescent leaves constitutes the majority of energy input into forested streams (Fisher and Likens 1973, Vannote et al. 1980). Due to resorption of nutrients by the trees prior to senescence, leaf litter is often very low in nutrient content (Aerts 1996, Vergutz et al. 2012) and thus food quality to macroinvertebrates.

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Microbes colonizing leaf litter immobilize dissolved inorganic nitrogen (DIN) and phosphorus (DIP) to allow growth and utilization of litter C pools (Suberkropp 1998, Gulis and Suberkropp 2003) Therefore, nutrient enrichment often results in detritus with a greater nutrient content (Cross et al. 2003, Scott et al. 2013) via increases in fungal and bacterial biomass (Suberkropp et al. 2010). However, these increases in detrital nutrient content may not always lead to improved food quality for consumers (Boersma and Elser 2006). Ecological stoichiometry (ES) theory, which studies the mass balances of multiple elements affect trophic interactions (Sterner and Elser 2002), suggests that nutrient elements ingested in excess of consumer demand may lead to C-limitation of growth (Sterner and Elser 2002, Frost et al. 2006) resulting in a paradox of declining growth as nutrient content of food surpasses the optimal growth requirements (Boersma and Elser 2006). One of the difficulties in assessing the effect of enrichment-driven changes in detrital stoichiometry on macroinvertebrate detritivores is that changes in quality often coincide with changes in detrital quantity.

Studies have shown stimulation of microbial processes such as decomposition and respiration rates with increasing nutrient concentrations. Elwood et al. (1981) examined the effects of enrichment on leaf litter decomposition rate and microbial activity in a small, P-limited woodland stream in Tennessee. When compared to the upstream control reach, both enriched downstream reaches had significantly faster decomposition rates. In a long term nutrient enrichment study of a detritus based stream, Suberkropp et al. (2010) showed that when compared to a reference stream, an enriched stream exhibited an increase in fungal and bacterial productivity and microbial respiration rates. These responses were also associated with much faster decomposition rates, decreased leaf litter standing stock, and limited food-web production. These effects in tandem with enhanced leaf litter nutrient content via microbial uptake of water

column nutrients indicate that nutrient enrichment can alter microbial stoichiometry and thereby the elemental composition of leaf litter. These relationships between litter stoichiometry and microbial processes are rarely examined but are needed to enhance our understanding of litter decomposition and nutritional quality for macroinvertebrates, and, therefore higher trophic levels.

Relationships between litter stoichiometry and microbial processes likely depend on initial or intrinsic litter characteristics that vary across tree species. Leaves vary in quality based on factors such as nutrient concentrations and lignin content. Differences in leaf litter C: nutrient ratios and differences in C quality between litter species represent differences in initial litter quality which can impact microbial activity, detrital processing, and decomposition rates in streams (Webster and Benfield 1986, Ardón and Pringle 2006). Litter quality can be described in terms of C: nutrient (N and P) ratios and C quality, both of which can vary based on litter species. Litter C is often categorized as labile or recalcitrant. Labile C molecules, such as cellulose, are small, easily broken down, and have high energy content, whereas recalcitrant C compounds, such as lignin, are larger, more complex molecules that are more difficult to break down and therefore provide less net energy (Sinsabaugh et al. 1993). Webster and Benfield (1986) compiled data from decomposition studies, showing differences in litter breakdown rates between different tree families, where families with higher percentages of recalcitrant lignin and lower initial nutrient content decomposed more slowly. This likely reflects difference in food quality for microorganisms and their ability to colonize and breakdown the litter material. Therefore, differences in surrounding plant species in riparian zones may affect microbial colonization and decomposition rates of allochthonous leaf litter (Webster and Benfield 1986). A dose-response relationship between nutrient concentrations and detrital stoichiometry and

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decomposition is needed to begin to determine at what nutrient levels litter quality and quantity may shift and mediate a change in detrital food webs. Further, we need to assess variation in this dose-response relationship associated with detritus originating from different tree species.

The objective of my study was to determine the effects of P enrichment and litter type on microbial-mediated decomposition rates and leaf litter stoichiometry. Two leaf species, sugar maple (*Acer saccharum*) and post oak (*Quercus stellata*), were chosen based on known differences in chemical compositions. Oak and maple forests dominate the study area and have distinctly different decomposition rates (Webster and Benfield 1986), which can be attributed to said differences in chemical properties of the litter material. I hypothesized the litter C:P would decline with time and then stabilize at some minimum value within each DIP concentration. Further, the minimum litter C:P observed over time would decrease with increasing DIP enrichment and decomposition rates would increase with increasing P enrichment in a saturating manner across the DIP gradient. I predicted that these effects would occur for both litter species, but with maple litter reaching a minimum C:P and maximum decomposition rate at a lower DIP concentration than on the more recalcitrant oak leaves due to differences in C availability between the litter species. Understanding these effects in detrital based ecosystems can enhance our understanding of how nutrient enrichment can affect nutritional quality and quantity of leaf litter, and therefore educate predictions about nutrient effects on higher trophic levels.

C. Methods

A 210 day laboratory microcosm experiment examined the effect of litter-litter type and increased dissolved inorganic P (DIP) concentration on microbial decomposition rates and leaf litter stoichiometry. Two litter types [recalcitrant oak (*Quercus stellata*) and labile maple (*Acer saccharum*)] were subjected to ten DIP treatments (0, 10, 25, 50, 75, 100, 250, 500, 1000, and

2000 µg/L added P as Na_2PO_4) resulting in 20 treatment combinations. Maple and oak material contained in separate bags were incubated together in three replicate microcosms at each DIP level (30 microcosms; 60 litter bags).

Recently-senesced leaves were collected in Washington County, Arkansas (AR), USA, air-dried at ambient temperatures, and stored in bags. Leaves were cut into 13.5 mm diameter disks, with major veins avoided, and stored at 48ºC prior to the start of the experiment. On Day 0 of the experiment 128 maple and oak disks were added to each replicate polypropylene mesh bag (10mm mesh/12cm*9cm) (Rashel Wicketed Baler Bags, Volm Companies, Antigo, WI) and placed in each replicate microcosm for each treatment. Additional decomposition bags (1mm/ 35cm*12cm) containing nine pre-weighed sets of three disks for each litter species were also placed in each microcosm and were destructively sampled over time.

Microcosms (1-L glass beakers) were filled with stream water from Jones Creek, a small 3rd order stream near Winslow, AR, USA to inoculate the leaves with aquatic microorganisms. Dissolved inorganic phosphorus additions equivalent to each of the DIP treatments were added accordingly. Nitrogen (N) as KNO_3 was added at 1 mg N/L to avoid potential microbial N-limitation. After day seven, the triplicate beakers were filled with dechlorinated tap water and again were spiked to one of the ten experimental DIP concentrations. Water was dechlorinated by allowing it to stand for at least 72 hours, open to the laboratory air. Complete dissipation of chlorine gas from the water was assumed. Microcosms were flushed with fresh stream water every two days for the first week and weekly with dechlorinated tap water for duration of the experiment. Appropriate DIP and DIN additions accompanied microcosm flushing.

Decomposition samples were harvested on days 7 and 14, then every four weeks for the remainder of the experiment (Days 7, 14, 42, 70, 98, 126, 154, 182, and 210). Dry mass was

determined by drying disks (48°C, $\geq$72 h) followed by weighing (nearest 0.0001 mg). Samples were combusted in a muffle furnace (Thermo Electron Corporation, Madison, WI; 550°C; 4 h) and reweighed to determine the ash-free drymass (AFDM).

Samples were harvested every two weeks to determine litter stoichiometry. Litter samples were dried at 48°C for at least 72 hours, and then ground up using a Wig-L-Bug grinding mill (Crescent Dental, Elgin, Illinois) to homogenize the sample. Litter material was weighed and combusted at 500°C prior to persulfate digestion. After digestion, total P was determined colorimetrically using the ascorbic acid method (APHA 2005) and analyzed using a Genesys 10vis Spectrophotometer (Thermo Scientific, Lakewood, NJ). Carbon was measured using a Thermo Flash 2000 Organic Elemental Analyzer (Thermo Scientific, Lakewood, NJ). Molar C:P ratios were calculated using these data.

Breakdown coefficients (k) were calculated for AFDM loss by regressing the ln (% mass remaining) in each beaker through time. Saturation of k (V_{max}) and the DIP concentration at which half-saturation occurred (K_m) were evaluated using Michaelis–Menten kinetic models (Sigma-Plot version 12.0; Systat Software, San Jose, California) because breakdown coefficients plotted across DIP treatments showed a saturating relationship. The Michaelis–Menten kinetics model was also used to determine the minimum C:P ratio for each DIP treatment over time. To fit the model, litter C:P ratios were inverted to produce a positive saturating relationship with time. This allowed estimation of the maximum P:C ratio at each DIP level. The mean litter C:P of all sampling dates was used to calculate this minimum C:P if no statistically significant relationship occurred between litter P:C and time. The Michaelis-Menten kinetics model was then used to determine the maximum P:C_{sat} value (V_{max}) across the DIP concentration gradient and the DIP concentration at which P:C half-saturates (K_m) for each litter species All P:C values

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were back calculated to C:P values for interpretation. Statistical methods are demonstrated in figure 1.

The saturating models were linearized using double-reciprocal plots of P concentration and the two variables, breakdown rate and minimum C:P values, to determine litter species effects using an analysis of covariance (ANCOVA); litter species was the categorical and DIP concentration was the covariate. ANCOVAs were performed using Systat (version 13; Systat Software, San Jose, California).

D.   Results

Leaf litter %P tended to increase through time and differ across DIP treatments, from 0.04-0.20 and 0.05 to 0.12 for maple (Figure 2) and oak (Figure 3), respectively. Leaf litter C:P decreased from approximately 3500 to 500 and from 3500 to 1000 for maple (Figure 4) and oak (Figure 5), respectively. The change in litter C:P is likely due to the changes in %P (Figure 2 and 3) rather than %C, which ranged from 46-58% . Leaf litter mean N:P ranged from 120-30 and 110-40 for maple (Figure 4) and oak (Figure 5), respectively.

The Michaelis–Menten kinetics model found statistically-significant relationships between maple and oak litter P:C and time at 25, 50, 75, 100, 250, 500, 1000, and 2000 µg P/L treatment levels (Table 1). The P:C_{sat} constant for each DIP treatment level was incorporated into the Michaelis-Menton relationship between litter P:C and DIP concentration (Table 2). Carbon:P in the 0 and 10 µg/L treatments for maple and oak did not change through time, so C:P values from all sampling days were used to calculate respective means within each microcosm for insertion into the relationship between litter P:C and DIP concentration. Along the gradient from 0 to 2000 µg/L P, C:P_{sat} values ranged from 3255 to 511 and 3373 to 870 for maple and oak litter, respectively. Litter P:C_{sat} values decreased in an inverse saturating relationship along the P

gradient (Figure 6). Maximum P:C_{sat} value (V_{max}) was lower for maple compared to oak litter, while the DIP concentration at which P:C reaches half-saturation (K_m) had overlapping standard errors suggesting no difference (Table 3). The ANCOVA showed no significant interaction effects (F=3.094; p>0.05). Minimum C:P values differed between litter types after accounting for DIP effects (Table 4).

Leaf litter decomposition rates as shown by breakdown coefficient (k) d^{-1}, increased in a saturating relationship along the DIP gradient (Figure 7). Along the gradient, values ranged from 0.0049 to 0.0087 and 0.0017 to 0.0030 for maple and oak litter, respectively (Table 2). Maximum litter decomposition rate (V_{max}) and the DIP concentration at which decomposition rate half-saturates (K_m) tended to be higher for maple litter compared to oak litter (Table 2). The ANCOVA showed no significant interaction effects (F=0.296; p>0.05). Leaf decomposition rates differed between litter types after accounting for DIP effects (Table 4).

E.   Discussion and Conclusions

The objective of my study was to determine the effects of P enrichment and litter type on litter stoichiometry and microbial-mediated decomposition rates. We found that increasing the availability of DIP led to increased litter quality by decreasing C:P ratios, with results indicating a lesser response of more recalcitrant litter types to nutrient enrichment. Results demonstrate an increase in leaf litter %P across an increasing DIP gradient, which led to a subsequent decrease in C:P and N:P for both litter types. Similar patterns in %P of litter with P enrichment have been shown (Elwood et al. 1981, Cross et al. 2003, Scott et al. 2013). Scott et al. 2013 saw increases in %P for maple and oak litter ranging from 0.05 to 0.25 and 0.05 to 0.15 for maple and oak across a P treatment range of 0 to 500 µg/L, which are comparable to the ranges from 0.04-0.20 and 0.05 to 0.12 for maple and oak, respectively, of this study. Further, Scott et al. (2013) found

similar decreases in leaf C:P, ranging from 3500 to 500 and from 2000 to 1000 for maple and oak, respectively. These changes in %P and subsequent changes in C:P are further supported by reported C:P values of different litter types in field studies. Cross et al. (2003) reported mean C:P ranging from 3000 to 5000 of mixed leaf litter in enriched and reference reaches, respectively. Further, Small and Pringle (2010) reported C:P ranges from 500 to 3500 of mixed litter in tropical streams of varying P concentration. So, while my findings are similar to patterns previously shown, my study provides a large DIP-gradient allowing the development of a quantitative model to link water DIP concentrations to leaf C:P and decomposition.

Literature C:P values for leaves have been reported in the range of 500-3500 (Stelzer et al. 2003, Cross et al. 2003, Small and Pringle 2010, Scott et al. 2013), whereas invertebrate C:P values have been reported in the range of 100-900 (Evans-White et al. 2005), demonstrating a naturally occurring consumer-resource imbalance in aquatic ecosystems. To meet their nutritional demands, invertebrates preferentially feed on higher quality substrates, such as microbial colonized leaf litter (Swan and Palmer 2006, Kaushik and Hynes 1971). However, at increasing nutrient concentrations, organisms that normally feed on high C:P food or are very strictly homeostatic, can be negatively affected by decreases in food resource C:P, due to metabolic costs associated with releasing excess P (Boersma and Elser 2006). This may ultimately result in community assemblages shifting in their body stoichiometries and decreases in biodiversity due to reduction or extirpation of certain species unable to cope with lower C:P ratios (Boersma and Elser 2006, Evans-White et al. 2009).

Additionally, microbial communities contribute to litter decomposition. In undisturbed forested stream systems such as those found in my study region, microbial communities are often nutrient limited (Rosemond et al. 2002, Hladyz et al. 2009, Hill et al. 2010). Therefore, nutrient

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enrichment can result in increased microbial activity such as increased decomposition and respiration rates (Rosemond et al. 2002, Gulis and Superkropp 2003, Suberkropp et al. 2010), and changes in litter quality (Cross et al. 2003, Small and Pringle 2010, Scott et al. 2013). My study results indicate similar responses to phosphorus enrichment, suggesting microbial P-limitation.

Similar to the results of my stoichiometry measurements, decomposition of oak leaves appeared to respond less strongly to nutrient enrichment than did decomposition of maple leaves. Oak leaves appeared to have lower breakdown coefficients across nutrient concentrations and breakdown rates stopped increasing with increasing DIP-enrichment faster than did maple leaves. This likely relates to initial litter quality as previously mentioned. Microbes of leaves with labile C can become P-limited due to high C availability, and will therefore respond greater to P enrichment, whereas microbes on recalcitrant leaves can be C-limited and therefore will not respond as strongly (Ardon and Pringle 2007). This data corresponds with previous studies that have shown different breakdown rates for different litter species (Elwood et al. 1981, Robinson and Gessner 2000, Royer and Minshall 2001, Grattan and Suberkropp 2001, Gulis and Suberkropp 2003, Ardon and Pringle 2007). More rapid decomposition of leaf litter in nutrient rich aquatic systems could affect the availability of detrital food resources to aquatic detritivores, thereby affecting detritivore growth/production (Wallace et al. 1997, Eggert and Wallace 2003). My data provide further evidence that, as nutrient enrichment of streams continues to increase globally, differences in litter species inputs into streams is an important consideration in maintaining detrital standing stock and detritus-associated communities (Woodward et al. 2012).

My study looked at leaf litter stoichiometry and decomposition rates across a wide and encompassing gradient of DIP concentrations. Potential thresholds in responses of microbial

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processes to DIP enrichment have been alluded to previously (Rosemond et al. 2002). These thresholds are points at which increasing DIP enrichment will not elicit further response of the microbial processes. By including a large number and range of DIP concentrations and using the Michaelis-Menten model, I was able to calculate half saturation constants (K_m) of DIP concentrations limiting to microbial decomposition rates and changes in leaf litter quality. While leaf litter species differences were strong in regard to absolute saturating breakdown rate and C:P ratios, the DIP concentrations at which saturation occurred was similar for both litter species, further indicating the importance of litter type in stream processing. These data suggest that detrital dynamics and bottom-up effects on stream biota may differ greatly under similar nutrient enrichment conditions based on the type of inputs from riparian vegetation and should be considered in the development of nutrient criteria.

In addition to differences in the extent of response of the two litter types are differences in the sensitivity of litter decomposition and stoichiometry to DIP enrichment. Decomposition rates saturate at <25µg/L DIP for both litter types, while changes in litter quality occur up to approximately 80µg/L DIP. These observed differences are important when considering the effects of nutrient enrichment on detrital quality and quantity and subsequent effects on higher trophic levels and stream processes, and could be used to educate nutrient criteria in detritus based systems.

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G. Acknowledgments

H.   Tables

Table 1. Michaelis-Menten (M-M) statistics for P:C over time for each DIP treatment. P:C sat values included in subsequent M-M model (Figure 5).

P treatment (mg/L)	Maple			Oak		
	$P:C_{sat}$	K_d (day)	p-value	$P:C_{sat}$	K_d (day)	p-value
25	0.0006	25	<0.001	0.000475	21	<0.001
50	0.0009	55	<0.001	0.000604	32	<0.001
75	0.0017	95	<0.001	0.000837	37	<0.001
100	0.0014	65	<0.001	0.000932	39	<0.001
250	0.0018	35	<0.001	0.001185	39	<0.001
500	0.0019	42	<0.001	0.001268	32	<0.001
1000	0.0017	26	<0.001	0.001131	19	<0.001
2000	0.0020	37	<0.001	0.00115	15	<0.001

Table 2. Decomposition regression statistics used to determine breakdown coefficients in each experimental replicate bag over time.

P treatment (mg/L)	Replicate	Maple R^2	Maple p-value	Maple k (day^{-1})	Oak R^2	Oak p-value	Oak k (day^{-1})
0	1	0.85	<0.001	0.005	0.57	0.018	0.002
0	2	0.92	<0.001	0.005	0.77	<0.001	0.002
0	3	0.88	<0.001	0.005	0.77	<0.001	0.002
10	1	0.70	0.005	0.004	0.49	0.035	0.002
10	2	0.91	<0.001	0.005	0.83	<0.001	0.002
10	3	0.72	0.004	0.005	0.89	<0.001	0.003
25	1	0.83	<0.001	0.006	0.75	0.002	0.002
25	2	0.90	<0.001	0.005	0.76	0.002	0.002
25	3	0.82	<0.001	0.007	0.91	<0.001	0.003
50	1	0.79	<0.001	0.005	0.77	<0.001	0.002
50	2	0.90	<0.001	0.006	0.80	<0.001	0.003
50	3	0.86	<0.001	0.007	0.78	<0.001	0.002
75	1	0.90	<0.001	0.008	0.66	0.007	0.003
75	2	0.95	<0.001	0.008	0.69	0.005	0.002
75	3	0.88	<0.001	0.007	0.90	<0.001	0.003
100	1	0.92	<0.001	0.008	0.88	<0.001	0.003
100	2	0.76	0.002	0.007	0.99	<0.001	0.004
100	3	0.72	0.004	0.007	0.89	<0.001	0.003
250	1	0.92	<0.000	0.009	0.86	<0.001	0.003
250	2	0.86	<0.001	0.008	0.86	<0.001	0.003
250	3	0.82	<0.001	0.007	0.80	<0.001	0.002
500	1	0.94	<0.001	0.009	0.91	<0.001	0.003
500	2	0.91	<0.001	0.009	0.88	<0.001	0.003
500	3	0.92	<0.001	0.009	0.82	<0.001	0.003
1000	1	0.88	<0.001	0.009	0.83	<0.001	0.003
1000	2	0.96	<0.001	0.009	0.93	<0.001	0.003
1000	3	0.94	<0.001	0.008	0.78	<0.001	0.003
2000	1	0.91	<0.001	0.008	0.85	<0.001	0.003
2000	2	0.93	<0.001	0.011	0.84	<0.001	0.003
2000	3	0.87	<0.001	0.008	0.88	<0.001	0.003

Table 3. V_{max} ($\pm$ 1 SE) and K_m ($\pm$ 1 SE) values from Michaelis-Menten curve of molar P:C_{sat} (Figure 5) and leaf litter decay rates (Figure 6) across the P gradient . V_{max} refers to the maximum P:C value and decay rate reached before the curve saturates. K_m is the P concentration at which P:C and decay rate is half the maximum value.

	P:C			k d^{-1}	
	V_{max}	C:P_{sat}	K_m (μg/L P)	V_{max} (k d^{-1})	K_m (μg/L P)
Oak	0.0012 $\pm$ 0.0001	833	37.26 $\pm$ 10.87	0.0029 $\pm$ 0.0002	4.15 $\pm$ 2.60
Maple	0.0020 $\pm$ 0.0001	500	40.1150 $\pm$ 11.71	0.0085 $\pm$ 0.0005	10.59 $\pm$ 4.35

Table 4. Analysis of covariance (ANCOVA) results comparing maple and oak litter stoichiometry values and decomposition values across the DIP gradient

Parameter	Effect	F-value	p-value
C:P	DIP	31.258	<0.001
	Leaf Type	13.743	<0.001
	Type*DIP	3.094	0.100
k d^{-1}	DIP	42.372	<0.001
	Leaf Type	534.014	<0.001
	Type*DIP	0.296	0.589

I.   Figure legends

Figure 1. Flow diagram depicting the progression the statistical analyses listed in the statistical analyses section of the methods.

Figure 2. Percent C, N, and P $\pm$ 1SE of maple leaf litter through time.

Figure 3. Percent C, N, and P $\pm$ 1SE of oak leaf litter through time.

Figure 4. Mean molar C:N, N:P, and C:P $\pm$ 1SE of maple leaf litter through time.

Figure 5. Mean molar C:N, N:P, and C:P + 1SE of oak leaf litter through time.

Figure 6. Relationship between dissolved inorganic phosphorus (DIP) and Molar $P:C_{sat}$ values. Fitted curves are based on the Michaelis-Menten model kinetics.

Figure 7. Relationship between dissolved inorganic phosphorus (DIP) and litter decay rate (k) d^{-1}. Fitted curves are based on the Michaelis-Menten model kinetics.
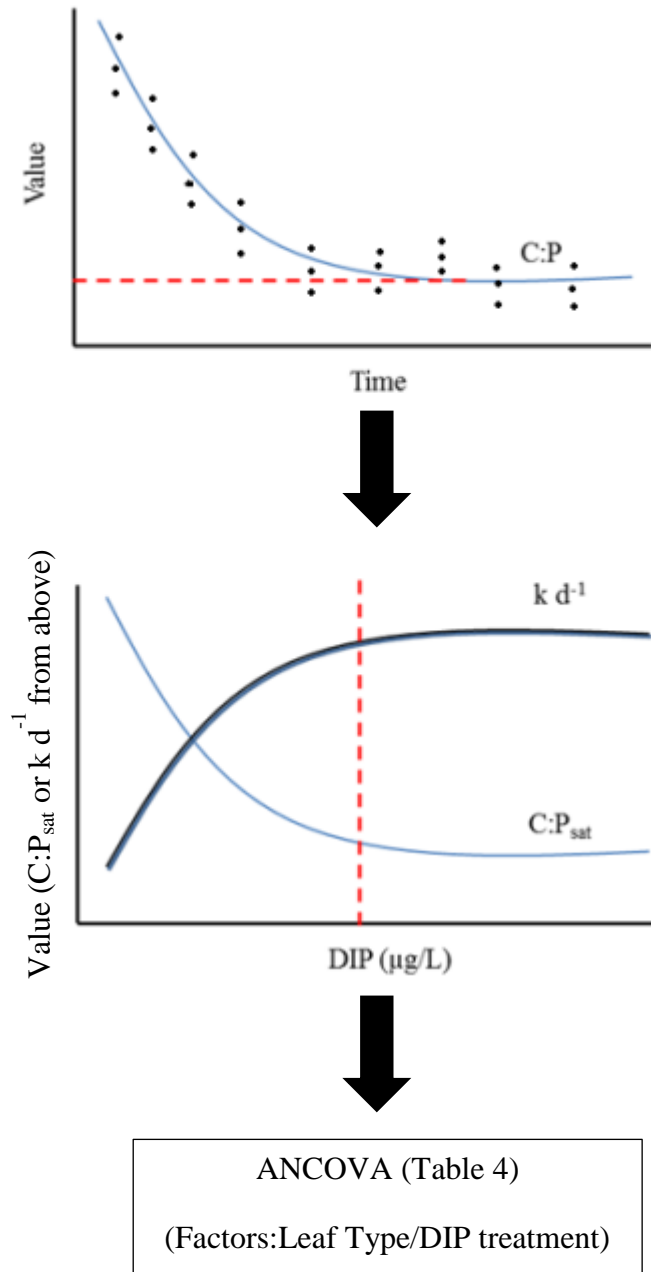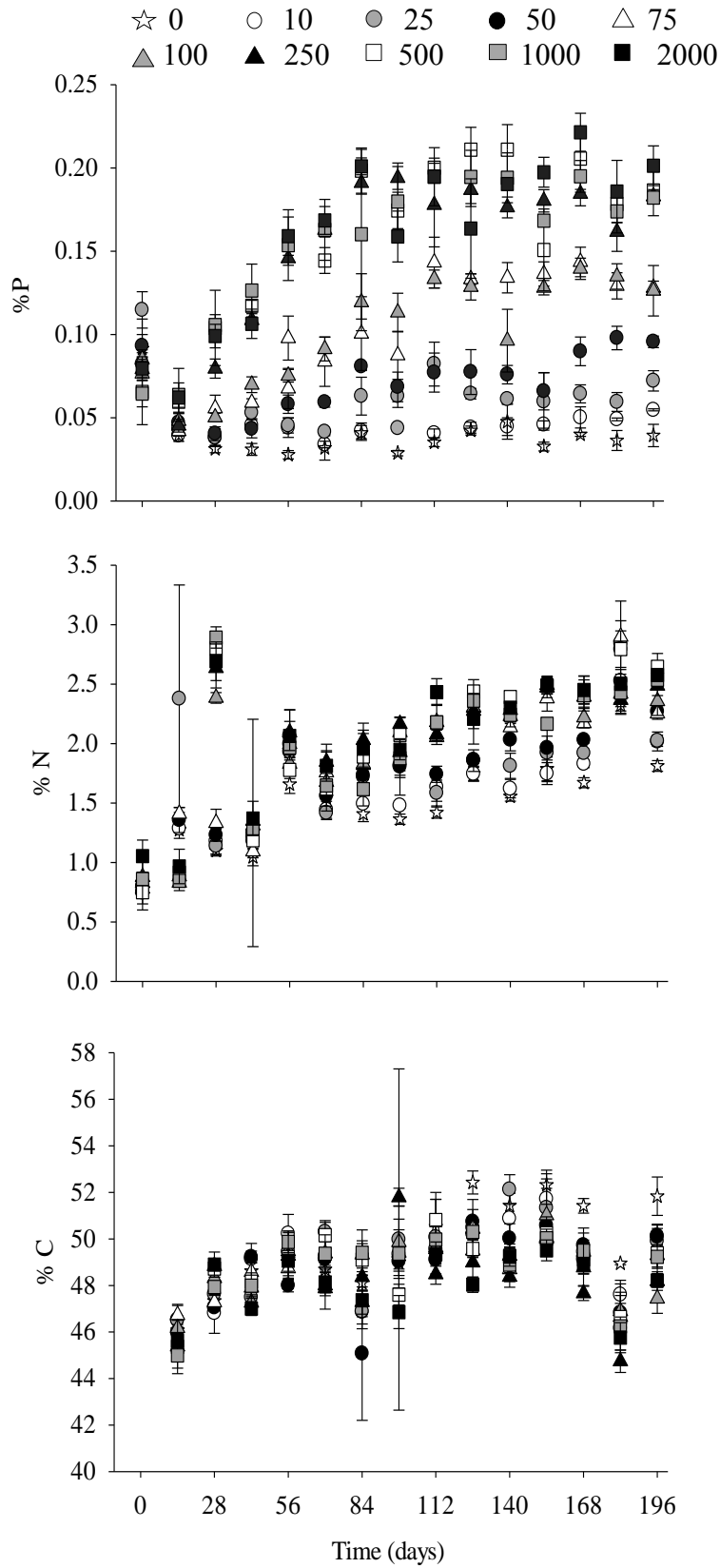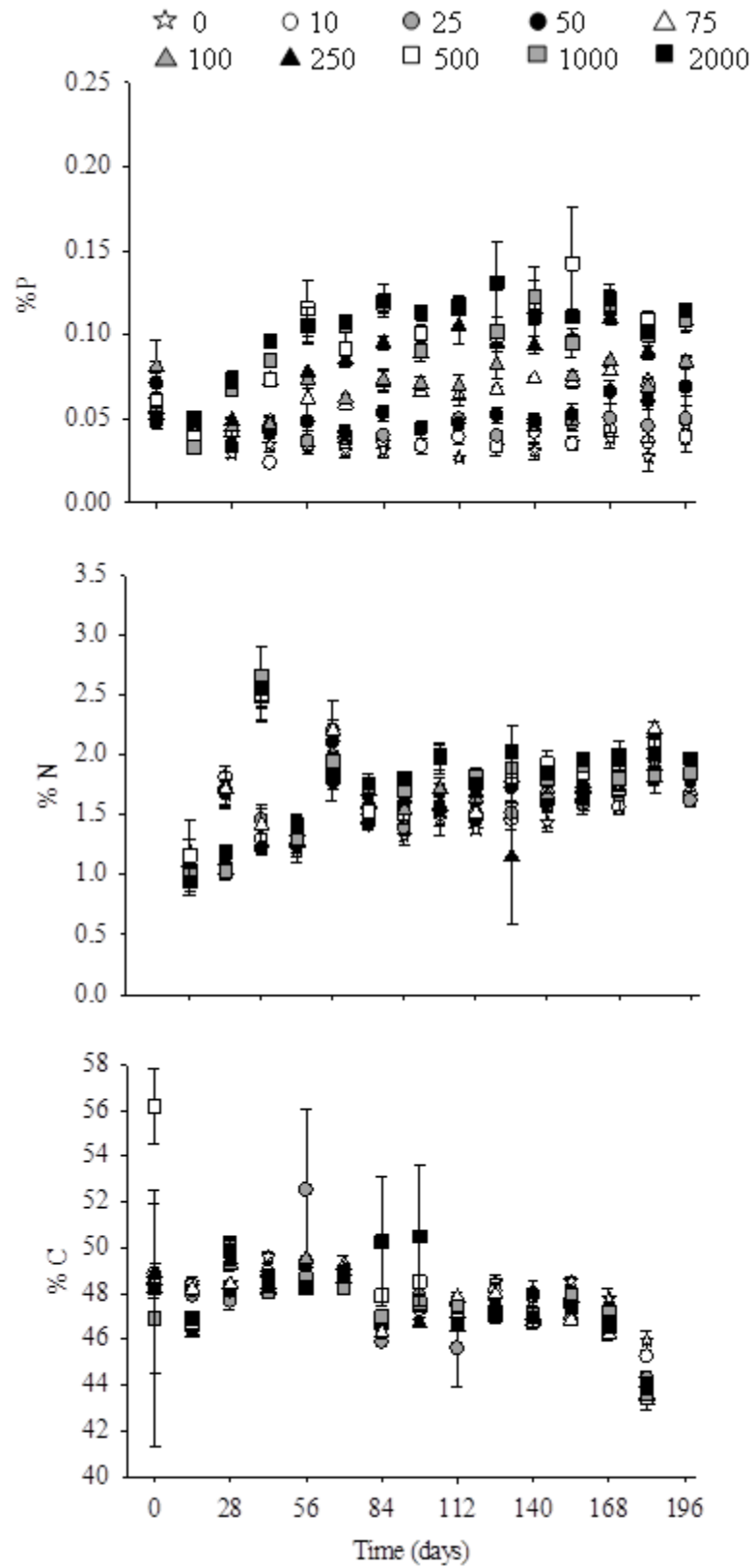
J.   Figures

Figure 1

Maple

Figure 2

Oak

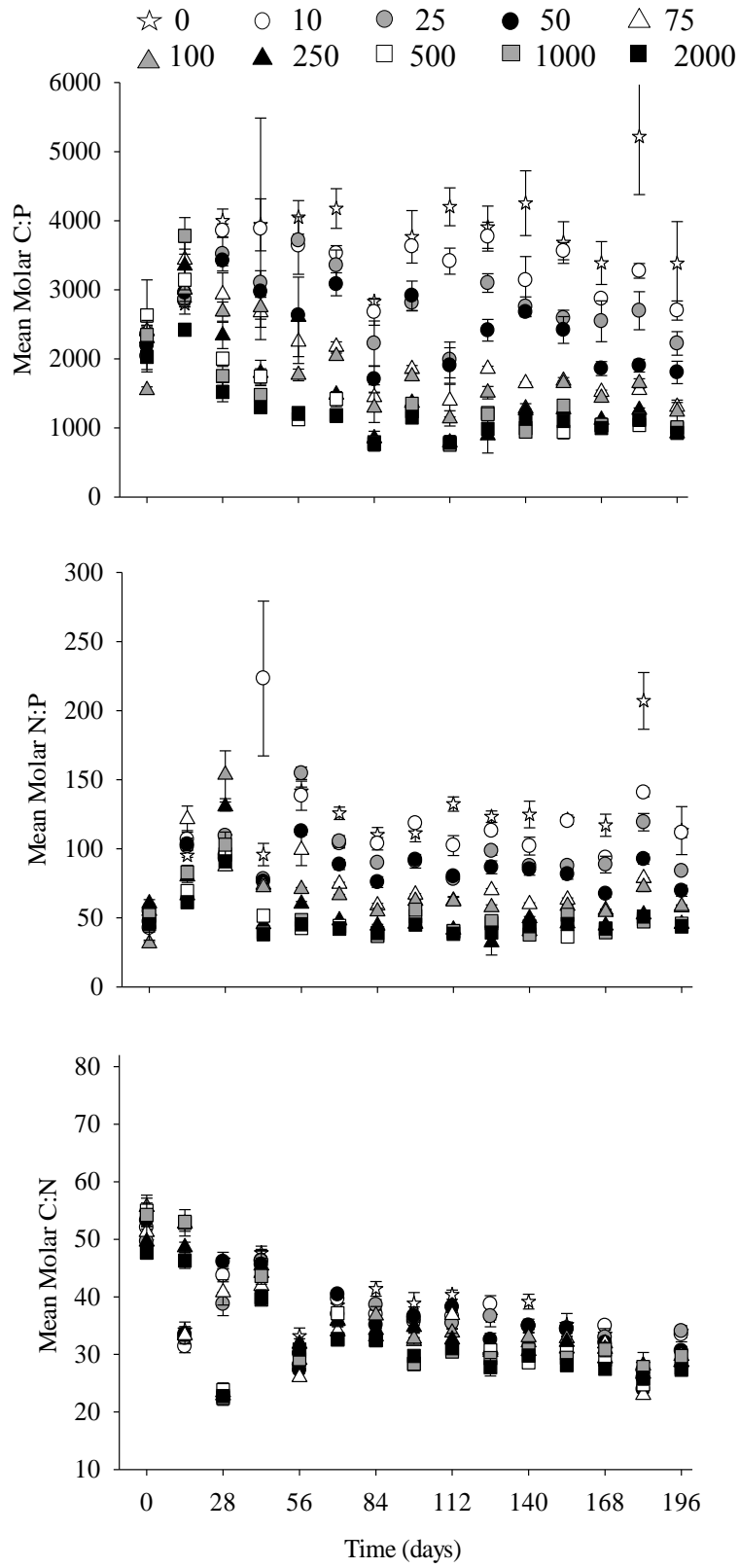**Figure 3**

Maple
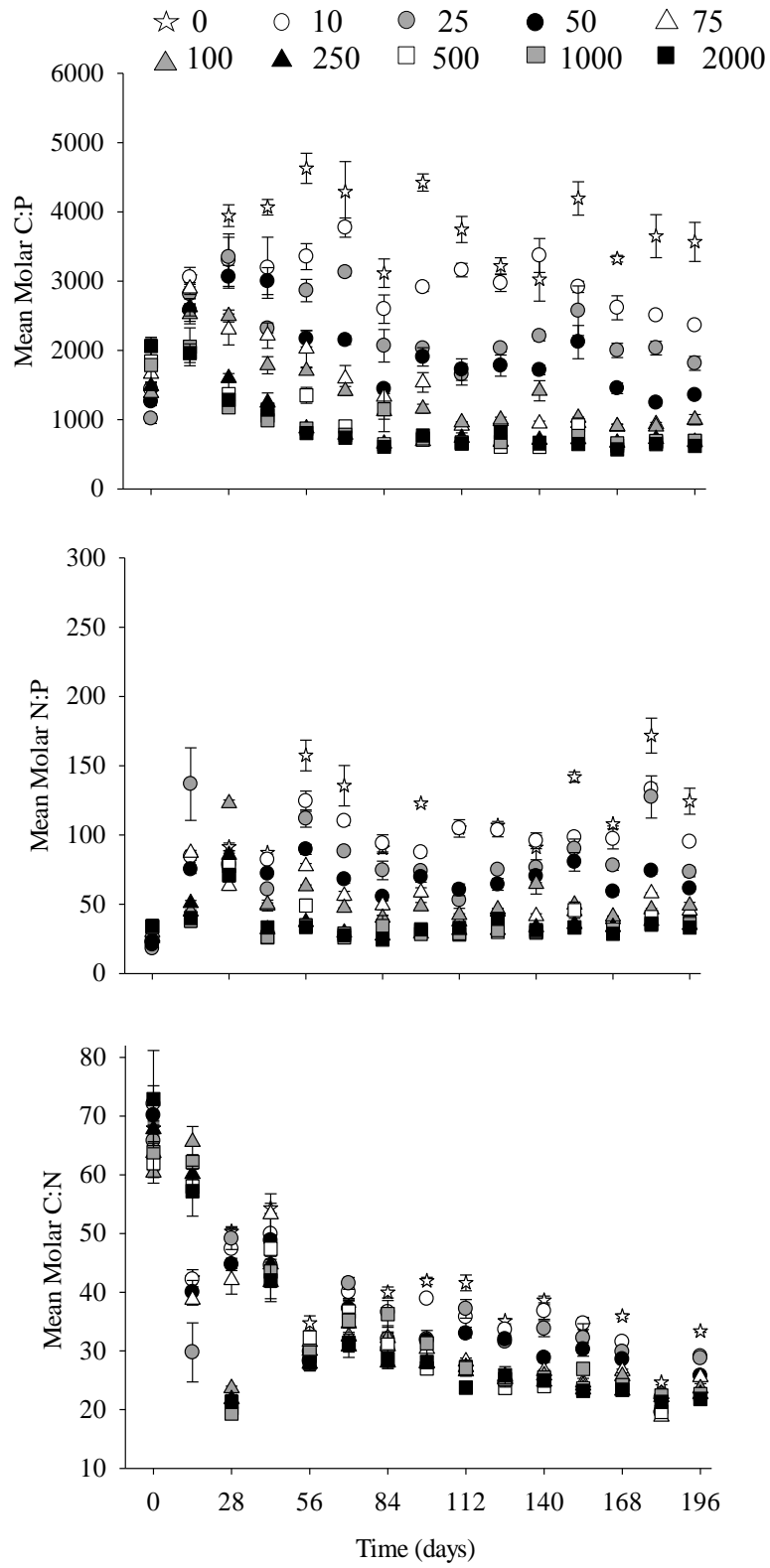
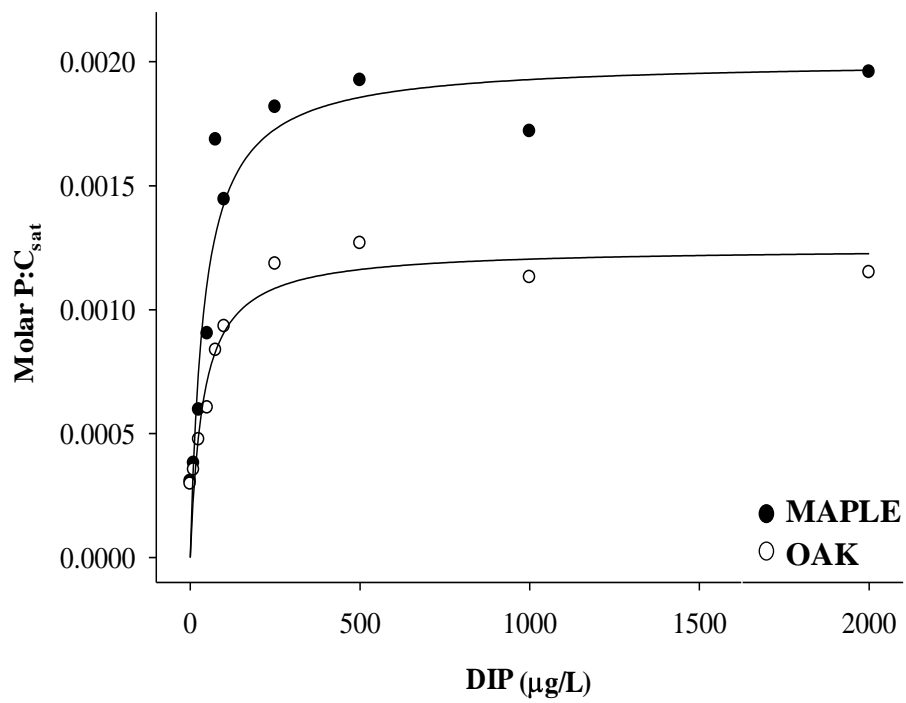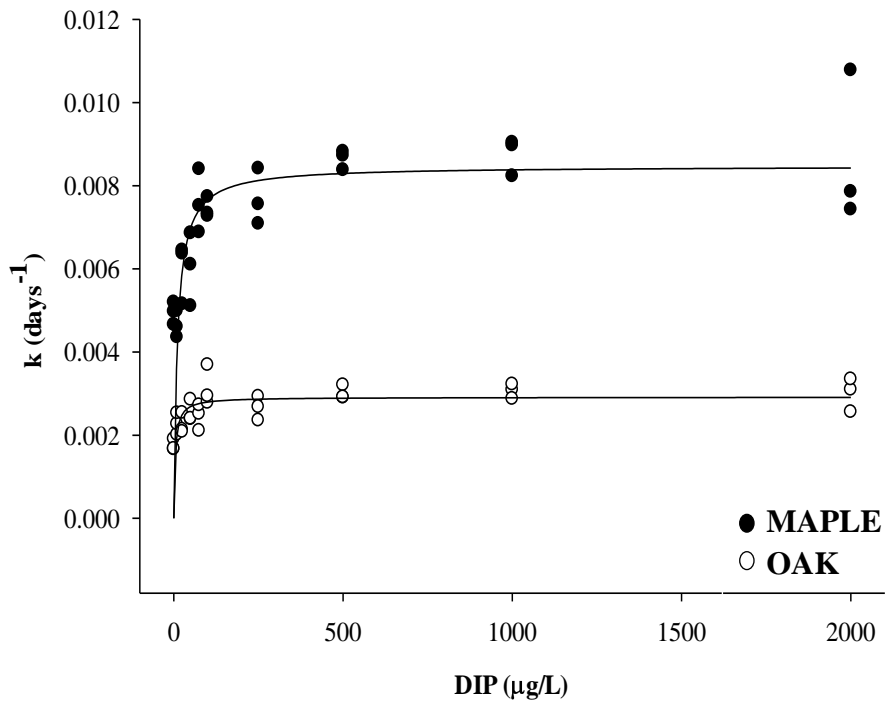

Figure 4

Oak

Figure 5

Figure 6

Figure 7

III. PATTERNS IN STREAM DETRITAL MICROBIAL P-LIMITATION OVER TIME WITH LITTER, STOICHIOMETRY, AND STREAM WATER PHOSPHORUS ENRICHMENT

A. Abstract

Decomposition measurements have been used widely as indicators of ecosystem functioning in streams. With increasing anthropogenic nutrient enrichment of streams, understanding relationships between stream structure and ecosystem function is important to help preserve ecosystem integrity. Development of quantitative relationships between decomposition rates and nutrient concentrations can provide information regarding nutrient limitation status. Decomposition however is time consuming to measure. We conducted a 210 day microcosm experiment to 1) examine the effects of time, litter type, and P-enrichment on respiration rates and alkaline phosphatase activity (APA) and to 2) determine whether these short term measures were related to litter stoichiometry and decomposition rates. Two-way repeated measures ANOVA showed a DIP*Day interaction ($F=4.07$, $p<0.001$) for APA and a Type*DIP*Day interaction ($F=1.51$, $p=0.027$) for respiration rates. Multiple comparisons revealed inconsistent trends in these data with the exception of consistent leaf type effects with maple having consistently higher rates when compared to oak. APA and C:P were positively correlated on day 28 for maple litter, and days 28, 56, and 168 for oak litter. Decomposition and respiration rates were weakly-positively correlated for oak litter on days 56 and 168. The data do not support the use of APA and respiration as instantaneous measure of limitation of decomposition in streams, but do provide insight into the complexity of the dynamics of nutrient enrichment in streams, and the necessity of multiple measures of stream structure and function to elucidate the effects of nutrient enrichment on stream ecosystems.

B.   Introduction

Microbial-mediated processes in streams are influenced by anthropogenic activities such as nutrient enrichment. Forested streams, which are predominant in the Ozarks, are often characterized by low water column nutrient concentrations, resulting in possible nutrient limitation of the biota (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), thereby reducing carbon (C) storage in stream ecosystems. In a long-term nutrient amendment study, enrichment resulted in decreased detrital standing stocks due to increased C export from streams via respiration and downstream transport (Benstead et al. 2009, Suberkropp et al. 2010). Enrichment-driven reductions in detrital C stocks in these streams may transfer up the food chain altering structure and production at many levels (Wallace et al 1997, Eggert and Wallace 2003). Further, this enrichment of detrital-based streams could coincide with threshold losses in biodiversity (Evans-White et al. 2009). As nutrient enrichment of streams and other ecosystems continues to increase globally, studies examining quantitative relationships between stream structure and ecosystem function become even more important to help guide management of these ecosystems and their services (Woodward et al. 2012, Palmer and Febria 2012).

Quantitative relationships between nutrient concentrations and decomposition would allow better predictions about nutrient-limitation of decomposition in streams. Further, this type of relationship could provide a nutrient concentration target area where threshold shifts in detrital-stream structure and function occur. However, decomposition measurements require several months of time and effort, making it difficult to broadly assess nutrient limitation of detrital processing. Snapshot measures of microbial respiration may be more feasible to examine broad spatial or temporal patterns in nutrient limitation of decomposition since increasing

microbial biomass (Rosemond et al. 2002, Benstead et al. 2005) and respiration rates (Greenwood et al. 2007, Suberkropp et al. 2010) have been associated with enrichment and greater detrital decomposition.

Microbial exoenzyme production may also provide short-term estimates of nutrient limitation of decomposition.   Studies have shown that microbial phosphatase activity responds to nutrient enrichment with increased activity under P-limited conditions (Healey and Hendzel 1980, Chrost and Overbeck 1987, Hill et al. 2006). Alkaline phosphatase is a non-specific, extracellular hydrolase enzyme that removes phosphate groups from dissolved organomolecules, making phosphates available for cellular assimilation. Determination of alkaline phosphatase activity (APA) is dependent on the enzymes ability to hydrolyze phosphate ester bonds, thereby releasing orthophosphate groups from organic phosphorous compounds. This hydrolysis reaction thus results in increased orthophosphate availability, as well as an increase in the abundance of the organic compound from which it is cleaved. APA is most commonly determined in aquatic systems by measuring the amount of organic compound byproduct produced after the phosphatase enzyme comes into contact with an added organic P substrate.

Alkaline phosphatase activity methods were initially developed for algal-associated alkaline phosphatase (Healey and Hendzel 1979) and are somewhat limited in aquatic ecosystems. APA has been used most widely in lake systems (Healey and Hendzel 1980, Rose and Axler 1998, Vrba 1995, Gao 2006) to assess periphyton and phytoplankton P-limitation. It has been less widely used in river systems, though methods have been successfully applied in wetlands (Scott et al 2007) and in marine ecosystems (Thingstadt et al.1998,). Cited studies suggest the successful use of APA as an indicator of P limitation on a multitude of aquatic

substrates including sediments, cobbles, and the water column, though APA has rarely been applied to leaf litter and heterotrophic microbial communities.

The use of nutrient ratios as indicators of nutrient limitation in aquatic systems is another technique that is most often applied to autotrophic communities and may be very limited in its applicability to heterotrophs. Autotrophs rely strictly on water column nutrients whereas heterotrophic biofilms found on detritus can utilize both water column nutrients and detrital nutrients (Gessner and Chauvet 1994). In this way, water column nutrient ratios do not indicate the entire pool of nutrients available to heterotrophic microbial communities, which may decouple the link between water column nutrient availability and nutrient limitation of heterotrophic biofilms. Compared to dissolved nutrient ratios, APA and microbial respiration rates are more direct responses of microbial communities to nutrient conditions, and are potentially more representative of nutrient limitation status. Therefore, understanding patterns in measurements of APA and respiration rates along a wide DIP gradient, in conjunction with litter stoichiometry data and decomposition data, may provide the possibility of more instant measures of P limitation status in detrital based systems and educate potential bottom up effects and management strategies.

The objective of the present study was to determine the effects of DIP enrichment and litter type on microbial community P limitation through analysis of APA and respiration rates over time. Additionally, my goal was to examine the relationship between APA and litter stoichiometry (Smartt et al. unpub.) and microbial respiration rates. I hypothesized that APA would be higher at lower P concentrations and would decrease for all DIP treatments over time, while respiration rates would increase across the DIP-gradient and through time. Further, I hypothesized that APA and respiration rates would be greater for maple leaves across the DIP

gradient and through time. Lastly, I hypothesized that APA and C:P ratios would be positively related and APA and respiration rates inversely related across the DIP gradient, while respiration rates would be positively related to decomposition rates. Understanding patterns in microbial-mediated processes and associated effects on detrital resources may educate predictions about nutrient effects on higher trophic levels. Further, understanding such patterns in measurements of APA and respiration rates along a wide DIP gradient may provide more instant measures of P enrichment in detrital based systems.

C.   Methods

To examine the effect of leaf-litter species and dissolved inorganic phosphorus (DIP) concentration on microbial respiration and APA production rates, a 210 day laboratory experiment was conducted. Two leaf litter types [recalcitrant oak (*Quercus stellata*) and labile maple (*Acer saccharum*)] were subjected to ten DIP treatment concentrations (0, 10, 25, 50, 75, 100, 250, 500, 1000, and 2000 µg/L added P as Na_2PO_4). Both litter types, contained in coarse mesh bags, were incubated together in three replicate microcosms of each DIP treatment (20 treatment combinations replicated three times; N= 60).

Recently-senesced leaves were collected in Washington County, AR and air-dried at ambient temperatures. Avoiding major veins, leaves were cut into 13.5 mm diameter disks and stored at 40ºC prior to the start of the experiment. To begin the experiment, 128 maple and oak disks were added to polypropylene mesh bags (10mm mesh /12cm*9cm) (Rashel Wicketed Baler Bags, Volm Companies, Antigo, WI) and placed in each replicate microcosm for each treatment. Decomposition bags (1mm/ 35cm*12cm) containing nine pre-weighed sets of three disks for each leaf species, to be destructively sampled over time, were also added to each replicate microcosm.

Microcosms (1L) were filled with 750 mL stream water from Jones Creek, a small 3rd

order stream near Winslow, Arkansas to introduce and inoculate leaves with aquatic

microorganisms. Phosphorus additions equivalent to each of the DIP treatments were added

accordingly and Nitrogen (N) as KNO_3 was added at 1 mg/L to avoid potential microbial N-

limitation. Microcosms were flushed on Day1, 3, and 5 with fresh stream water due to heavy

leaching. Nutrients were re-added as described. On Day 7, assuming inoculation of the leaf litter,

microcosms were instead flushed with dechlorinated tap water and were spiked to the

appropriate P concentrations. Microcosms were flushed weekly with dechlorinated tap water for

duration of the experiment. Appropriate DIP and N additions accompanied microcosm flushing.

Microbial respiration was measured as leaf disk oxygen uptake over time. Every four

weeks 1 leaf disk of each species was harvested from each microcosm and placed in 24 mL

scintillation vials. Vials were filled with dechlorinated tap water spiked to P concentrations

matching the given leaf disk treatment. An initial sample of each P treatment without a leaf disk

was preserved using 50% ZnCl to determine initial oxygen concentrations. Vials were capped,

leaving no head space, and allowed to incubate overnight, allowing respiration to occur and

oxygen to be consumed. Initial and final dissolved oxygen (DO) concentrations were measured

using a membrane inlet mass spectrometer (MIMS) (Kana et al. 1994). Oxygen consumption

rate was converted into a respiration rate following Wetzel and Likens (2000).

APA was measured using methods modified from Scott et al. (2009). Every four weeks a

single leaf disk from each chamber was submerged in a 24 mL scintillation vial with 14 mL of

dechlorinated tap water spiked with P to attain the concentration of the given nutrient treatment.

Three and one half mL of $0.2\mu M$ 4-methylumbelliferylphosphate (MUFP) was added to each

vial. Fluorescence was measured using a fluorometer (Turner Design Trilogy, Sunnvale, CA)

after 5, 25, and 60 minutes to determine rates of phosphate cleavage. APA was estimated using the rate by which phosphate is cleaved from MUFP, producing methylumbelliferone.

In addition to respiration rates and APA, decomposition and litter stoichiometry were also measured in this study. Decomposition samples were harvested on days 7 and 14, then every four weeks for the remainder of the experiment (Days 7, 14, 42, 70, 98, 126, 154, 182, and 210). Litter samples were collected every 2 weeks for duration of experiment to determine litter stoichiometry. Refer to Smartt (CH1) for full methods. Analysis of these data showed statistically significant M-M relationships between decomposition rates and DIP for both species indicating P-limitation of decomposition (Smartt, unpublished).

Effects of P treatment, litter type, and time on respiration rates and APA were analyzed with a 2-way repeated measures ANOVA using the MIXED procedure in SAS (ASA Institute Inc., Cary, NY, USA). Where significant, multiple comparisons were completed using the LSMEANS statement with a TUKEYS adjustment in SAS. Respiration rates and APA data were log(x)-transformed to meet the assumptions of the tests.

Relationships between APA and litter stoichiometry and respiration rates, and respiration rates and decomposition rates were analyzed using Spearman correlations. All data were log(x)-transformed. All correlations were performed using Systat statistical software (version 13; Systat Software, San Jose, California).

D.   Results

The two-way repeated measures ANOVA showed no interaction of Type*DIP*Day (F=1.23, p=0.168) on APA, but did show significant interactions of DIP*Day (F=4.07, p<0.001) (Table 1). Multiple comparisons showed significant differences (p<0.05) between concentration at each day, however consistent trends were shown except for day 28 and 56 (Figure 1). On these

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days APA decreased along the P gradient, ranging from 19.1 ± 3.6 to 3.6 ± 0.8 and 6.3 ± 1.2 to 0.3 ± 0.1 ng P_{ase}/mg AFDM/min, for each day respectively. Multiple comparisons showed no consistent trends within a concentration over time. Maple litter was statistically significantly higher than oak litter on all sample dates (Figure 2).

Two-way repeated measures ANOVA results on respiration rates found a significant interaction of Type*DIP*Day (F=1.51, p=0.027) (Table 1). Multiple comparisons showed statistically significant differences across the P gradient at each sample date, as well as through time for each DIP treatment, though no consistent significant trends were evident. Respiration rates on maple litter were consistently higher for maple leaves than for oak leaves (Figure 3).

APA and C:P were positively correlated on day 28 for maple litter, and days 28, 56, and 168 for oak litter, as hypothesized. No correlations were found between APA and respiration (Table 2) (Figures 4 and 5). Decomposition and respiration rates were correlated for oak litter on days 56 and 168 but not at all for maple litter (Table 3) (Figures 6 and 7).

E.   Discussion and Conclusions

 Decomposition rates measured over 210 days in the present study exhibited a saturating relationship with dissolved inorganic P indicating P-limitation (Chapter 1). The objective of this portion of the study was to determine if short-term measurements of APA and/or microbial respiration could be used as quick indicators of this decomposition response to P enrichment. My short-term measures of APA and respiration on leaf litter during the decomposition process yielded variability in the response over time and were not always indicative of P-limitation of decomposition.

The response of leaf disk APA to DIP enrichment was not consistent over time. Elevated APA, which would suggest P-limitation, was only evident below 250 µg/L DIP on days 28 and

56 in the conditioning process. Studies looking at APA in periphyton have shown responses of APA to nutrient enrichment at similar time frames as where my study found indication of P-limitation. In a study using flow-through artificial streams where ambient (14.2 $\pm$ 1.2 µg/L SRP) and enriched (264.8 $\pm$ 66.6 µg/L SRP) conditions were applied. APA, measured on day 28 of the experiment, was significantly higher under ambient conditions, when compared to enriched conditions, where less P-was available (Evans-White et al. 2006). An additional *in-situ* NDS study using APA as a measure of P-limitation found the same results under control and enriched conditions (Scott et al. 2009). These studies did not look at long term enrichment effects on APA expression where it may not be as strong of an indicator of P-limitation. This may play an important role in using APA as an indicator of P-limitation, where measurements are required within a short timeframe after microbial conditioning to accurately depict nutrient limitation of decomposition. Decomposition is a lengthy process and processes that resulted in the positive saturating relationship between DIP and decomposition (see Chapter 1) need not be constant over time. Therefore, short-term point measurements of APA did not always accurately predict P-limitation of leaves.

When litter C:P reaches a certain level, microbial communities are no longer P-limited. Positive correlations between APA and litter C:P ratios in addition to decreases in C:P over time (Chapter 1) suggest that APA was negatively related to microbial P content. Microbes should release less AP if P is in excess or being stored (i.e., luxury consumption). I did not measure microbial cell abundance in this study, so it is not clear whether the increase in P content we see with P enrichment and time is due to greater cell abundance or greater cell P content. Differences in homeostasis of microbes found on the leaf litter in response to enrichment can affect this dynamic (Scott et al. 2012, Danger 2013). Scott et al (2012) conducted a chemostat

experiment looking at the 6 different bacterial isolates across a C:P gradient, finding that bacteria may differentially increase uptake (non-homeostasis) or increase biomass (homeostasis) when P conditions are sufficient depending on strain. Danger (2013) further suggests that fungal non-homeostasis should be considered in stoichiometric models and that differences in the behaviors of different strains of microbes is also important. Fungal and bacterial biomass have, however, been shown to increase with nutrient enrichment (Robinson and Gessner 2000, Grattan and Suberkropp 2001, Gulis and Suberkropp 2003). Consistency of this pattern between APA and litter C:P would have to be confirmed before considering APA as an indicator for P-limitation status of detritus.

Short-term respiration incubations showed some indication of P-limitation at low DIP concentrations, but the relationship was not statistically significant and did not hold through time. Positive correlations between respiration and decomposition could potentially point to the use of respiration as a surrogate for decomposition in determining P-enrichment; however these relationships explained very little variation in decomposition. Further studies examining patterns in respiration over time along with complete mass loss under ambient and enriched nutrient conditions to determine if there is a time-frame, possibly early in the decomposition process as seen in the present study, that would more accurately predict nutrient limitation of decomposition. These studies would provide a greater level of confidence in the use of respiration as an indicator of heterotrophic nutrient limitation status given the variability in respiration rates measured in this study over time and the weak relationship found between respiration and decomposition rates.

The variability in short term microbial process measurements could be due to changes in the microbial community and nutrient use over time. Leaf litter associated microbial

communities undergo periods of growth and death during the decomposition process. These microbial communities are able to utilize nutrients from both the water column and leaf litter itself (Suberkropp 1998, Gulis and Suberkropp 2003). These nutrients may be cycled through the biofilm in different ways based on current microbial community dynamics. Cheever et al (2013) also provided evidence for differential use of water column and leaf nitrogen (N) by microbial communities over time. Likely, decomposition rates integrate these dynamics over time more accurately than short-term microbial process measurements.

Strong leaf type effects on APA and respiration were shown, indicating the importance of leaf type on microbial dynamics and responses to nutrient enrichment in streams. Specifically, my study indicated that the microbial communities on more labile maple leaves responded more strongly to P enrichment than on relatively recalcitrant oak leaves. Similar results have been observed for other microbial processes (Hladyz et al. 2009). Ardón and Pringle (2007) showed 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 microbial 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. Based on these relationships, it is likely that lower APA and respiration rates on oak leaves that were observed in my study was a result of C-limitation, due to less labile C available to microbes on oak compared to maple litter. When C becomes limiting, it would no longer be metabolically favorable to produce and exude alkaline phosphatase, regardless of potential P-limiting conditions, and respiration rates would be restricted by C and not respond to further P-additions.

Based on my findings it could be argued that leaf type will be more important than P concentration or time when potential effects of enrichment on an aquatic ecosystem and enrichment threshold values are being considered. There is, however, the potentially confounding effect of normalizing these measurements by AFDM. Differences in the masses of the two leaf types, and the inability to differentiate between leaf mass and dead and living microbial biomass, may impact the overall calculations of microbial activities. Differences appear less strong when measurements were normalized by area.

Aside from strong leaf effects, the results of this study do not support the use of APA or respiration as consistent indicators of the state of microbial P-limitation in detrital-based aquatic ecosystems. Though the use of APA as an instantaneous indicator of P-limitation was not strongly supported here, this option could be explored more thoroughly in detrital-based systems. Other aquatic studies looking at APA and other methods to indicate P-limitation have found that P deficiency indicators do not consistently indicate P deficiency and suggest the need to use multiple nutrient deficiency indicators when assessing the nutrient status aquatic systems. (Jamet et al., 2001, Cao et al., 2010,Vandergucht 2013). Similarly, biofilm respiration rates are often measured in streams to assess nutrient limitation (Tank et al 1993, Ramirez et al 2003, Tank and Dodds 2003, Stelzer et al 2003, Suberkropp et al 2010, Dempsey et al 2010). These values may be more indicative of the status of the microbes present at the time of sampling rather than the state of limitation through time as is seen with decomposition studies. Because microbial communities and processes and their responses to nutrient limitation appear dynamic over time, caution should be taken when using these potential indicators of limitation because scientists and managers may inaccurately determine nutrient limitation is not present for heterotrophic processes. It may also cause errors in determining the nutrient concentrations where limitation of

heterotrophic processes are relevant, which would make incorporation of heterotrophic processes into nutrient criteria development problematic (Evans-White et al. 2013)

Decomposition measurements have been cited as a prime way to assess stream ecosystem functioning (Gessner and Chauvet 2002). A widespread European study looking at 100 different streams spanning 1000-fold differences in nutrient concentrations used litter bags to measure differences in decomposition rates (Woodward et al. 2012). They found that when macroinvertebrates were not excluded, decomposition rates were highest at mid-level concentrations and similar at low and high-levels, providing insight into the dynamic nature of stream functional responses to nutrient enrichment. Tiegs (2013) has used a cotton-strip assay to look at decomposition in streams, showing a significant but weak relationship between ammonium and SRP and tensil-strength ($R^2 = 0.11$, $p = 0.023$; $R^2 = 0.08$, $p = 0.046$, respectively). This result could be attributed to low nutrient concentrations (19-104 µg/L ammonium and <5-48 µg/L SRP) across the gradient of streams used. Functional responses measured in the study suggest these methods may be a strong and more uniform alternative to assess enrichment effects on heterotrophic processes for management of aquatic ecosystems.

Of the indicators discussed, none is all encompassing in understanding the effects of nutrient enrichment on stream functioning. In order to assess stream integrity, measures of stream function should be coupled with measures of stream structure and already established bioassessment techniques to evaluate effects of enrichment and educate the development of nutrient criteria. While measurements taken in my study don't necessarily support their use as instantaneous indicators of limitation, the complex dynamics suggest further studies of microbial responses to enrichment in detrital based systems are needed.

F.   References

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F.   Acknowledgements

Table 1. Repeated measures two-way analysis of variance tables for microbial responses to DIP enrichment over time. Signficant effects that were investigates are highlighted in grey.

Parameter	Effect	P-value
Respiration	Litter type (Type)	<0.001
	DIP	<0.001
	Type*DIP	0.647
	DIP*Day	<0.001
	Type*Day	0.193
	Type*DIP*Day	0.027
APA	Litter type (Type)	<0.001
	DIP	<0.001
	Type*DIP	0.069
	DIP*Day	<0.001
	Type*Day	<0.055
	Type*DIP*Day	0.168

Table 2. Spearman correlations between APA and leaf stoichiometry and respiration rates for each sample date (correlation coefficient (r), probability (p)-value. Cells highlighted in gray show statistically significant relationships. Bonferroni corrected, $p<0.008$.

Day	Maple		Oak	
	C:P	Respiration	C:P	Respiration
	r, p	r, p	r, p	r, p
28	0.670, <0.001	-0.307, 0.098	0.658, <0.001	-0.155, 0.411
56	0.366, 0.074	0.155, 0.418	0.637, <0.001	-0.356, 0.058
84	0.357, 0.053	-0.034, 0.859	0.277, 0.144	0.009, 0.961
112	0.340, 0.088	-0.336, 0.086	0.468, 0.014	-0.372, 0.055
140	0.362, 0.049	0.120, 0.520	0.135, 0.474	0.170, 0.367
168	-0.251, 0.187	0.338, 0.090	0.586, <0.001	0.059, 0.756

Table 3. Spearman correlations between respiration rates and final decomposition breakdown coefficients for each sample date (correlation coefficient (r), probability (p)-value. Cells highlighted in gray show statistically significant relationships. Bonferroni corrected, $p < 0.008$.

	Maple	Oak
Day	r, p	r,p
28	0.370, 0.004	0.431, 0.018
56	0.370, 0.044	0.575, <0.001
84	0.265, 0.164	0.275, 0.147
112	0.396, 0.030	0.359, 0.050
140	0.474, 0.008	0.369, 0.044
168	0.116, 0.416	0.494, 0.006

H.   Figure legends

Figure 1. Mean $\pm$ 1 SE APA for each P concentration with litter type averaged. Within each date, different letters represent statistically significant difference between concentrations.

Figure 2. Mean $\pm$ 1 SE APA and respiration rates of all P concentrations for maple and oak litter at each sample date.

Figure 3. Mean $\pm$ 1 SE respiration rates of two leaf types for each P concentration at each sample date. Asterisks indicate significant differences between leaf types at a given concentration.

Figure 4. Relationship between leaf litter C:P and APA along the P gradient for maple litter at each sample date. Spearman correlation coefficients are shown in Table 2.

Figure 5. Relationship between leaf litter C:P and APA along the P gradient for oak litter at each sample date. Spearman correlation coefficients are shown in Table 2.

Figure 6. Relationship between leaf litter respiration rates and decomposition along the P gradient for maple litter at each sample date. Spearman correlation coefficients are shown in Table 3.

Figure 7. Relationship between leaf litter respiration rates and decomposition along the P gradient for oak litter at each sample date. Spearman correlation coefficients are shown in Table 3.
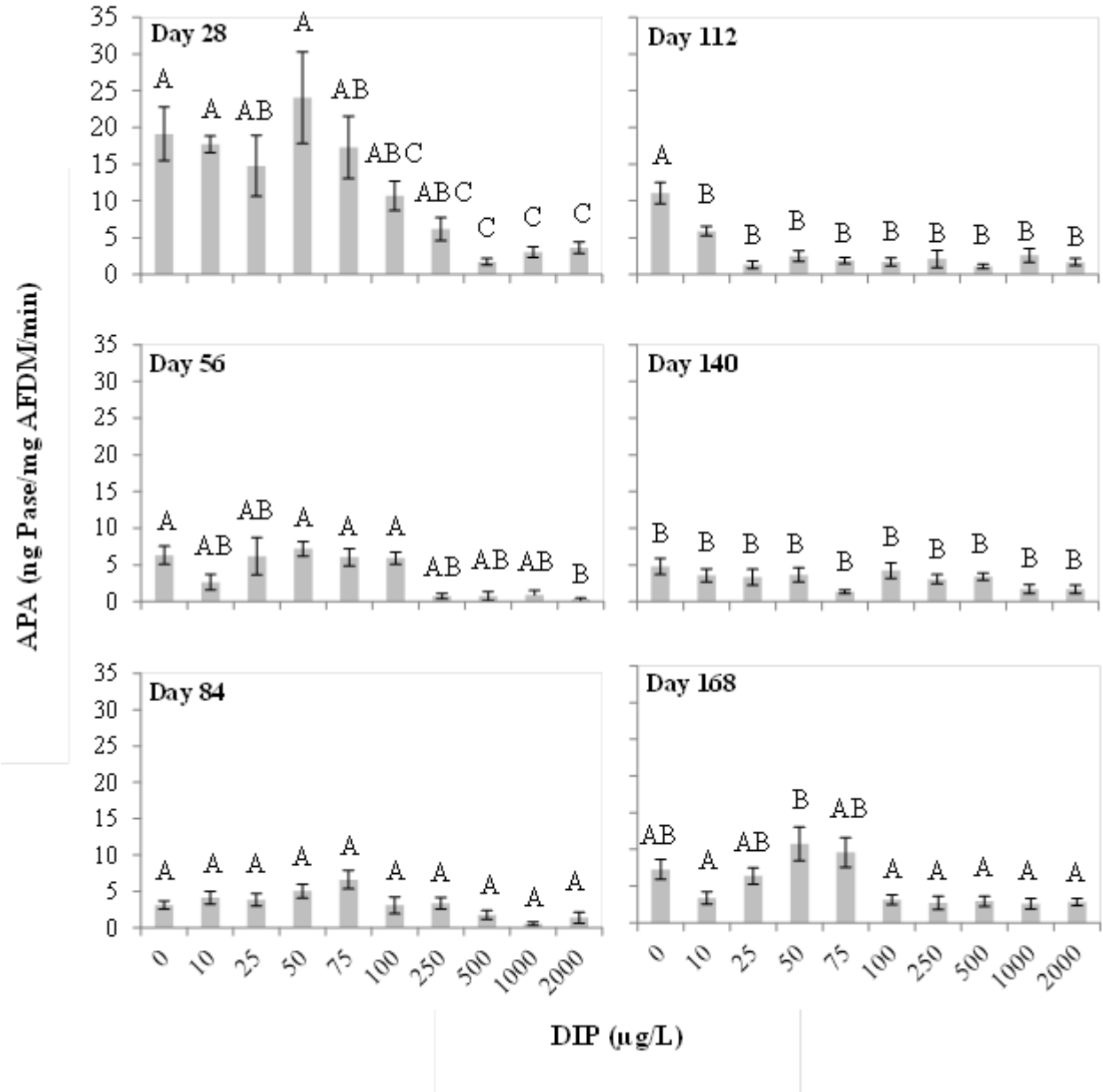
Figure 1

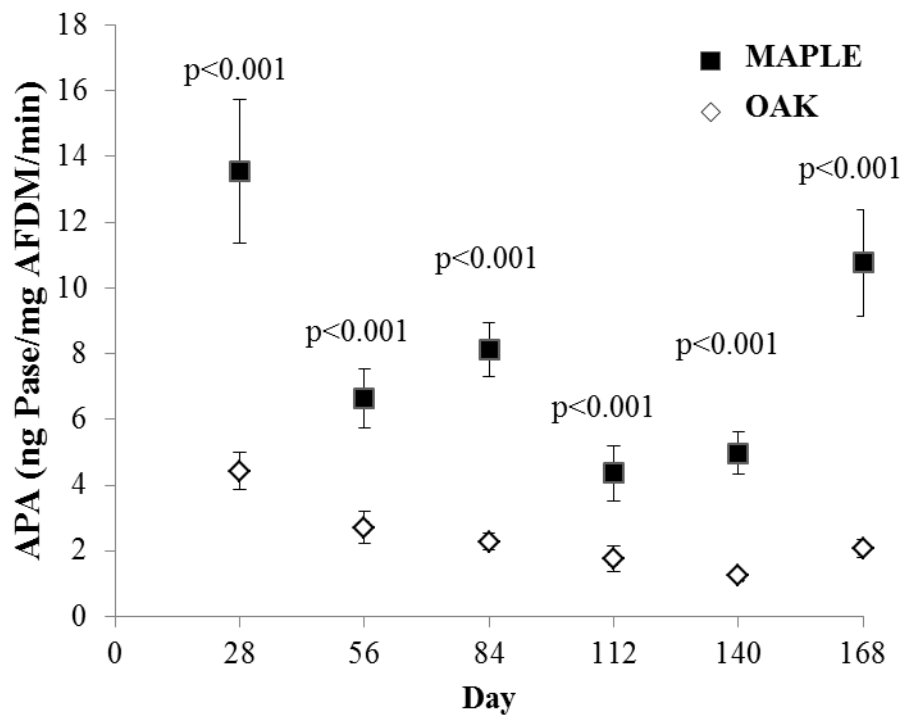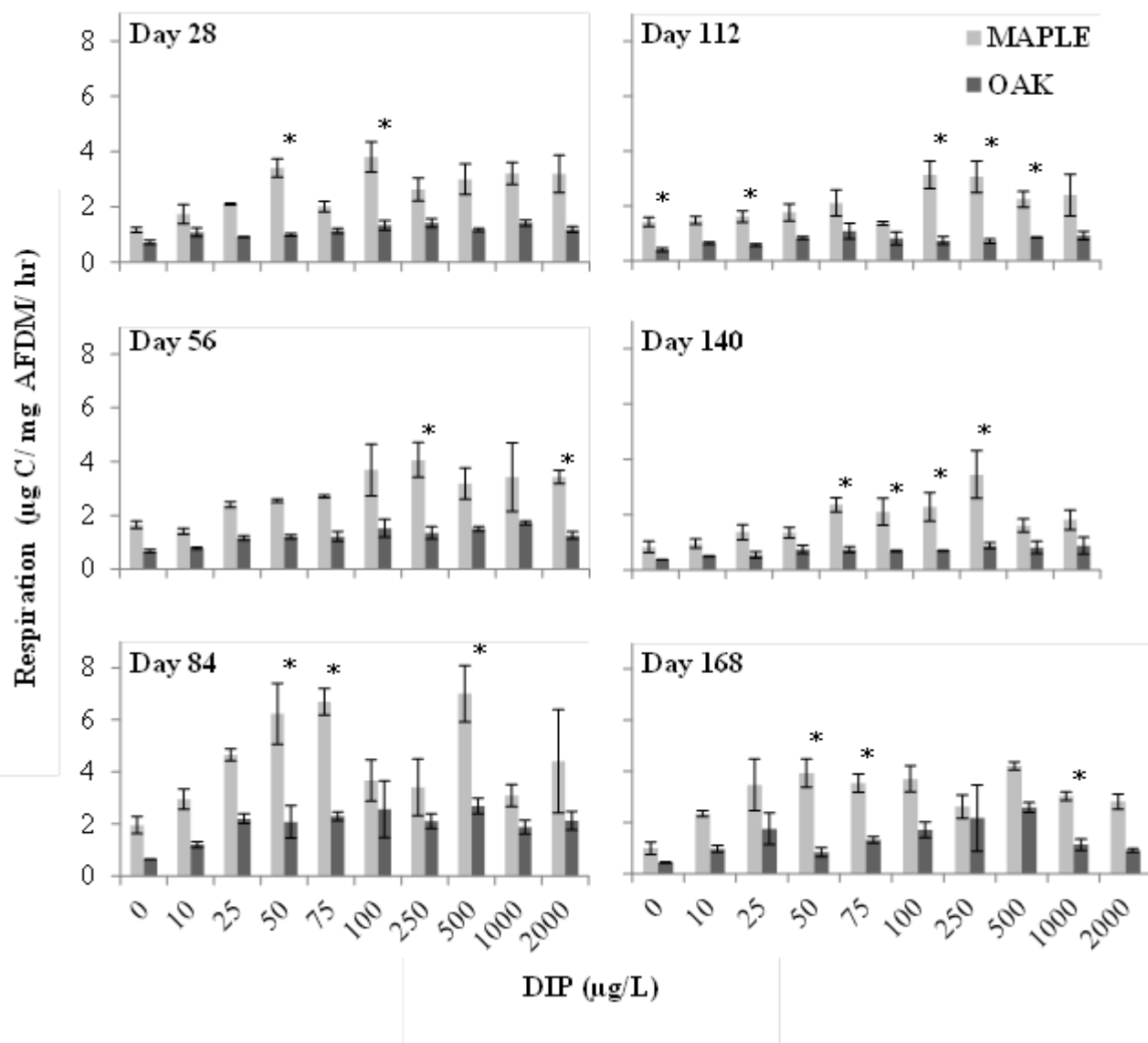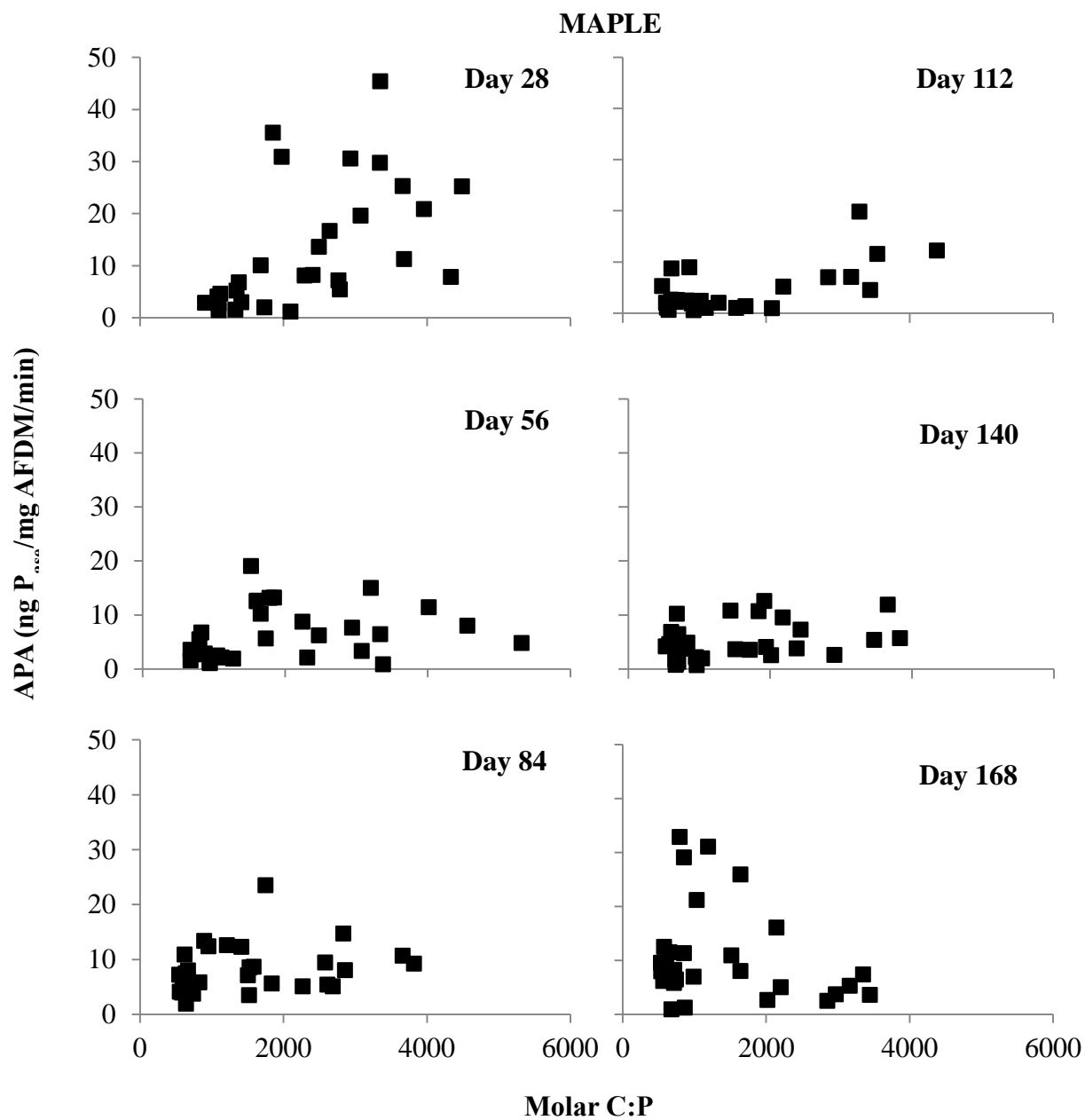**Figure 2**

Figure 3

MAPLE

APA (ng P$_{ase}$/mg AFDM/min)

Molar C:P

Day 28
Day 112
Day 56
Day 140
Day 84
Day 168

Figure 4

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OAK

Figure 5

Figure 6

OAK

Respiration (ug C/ mg AFDM/ hr)

K (days^{-1})
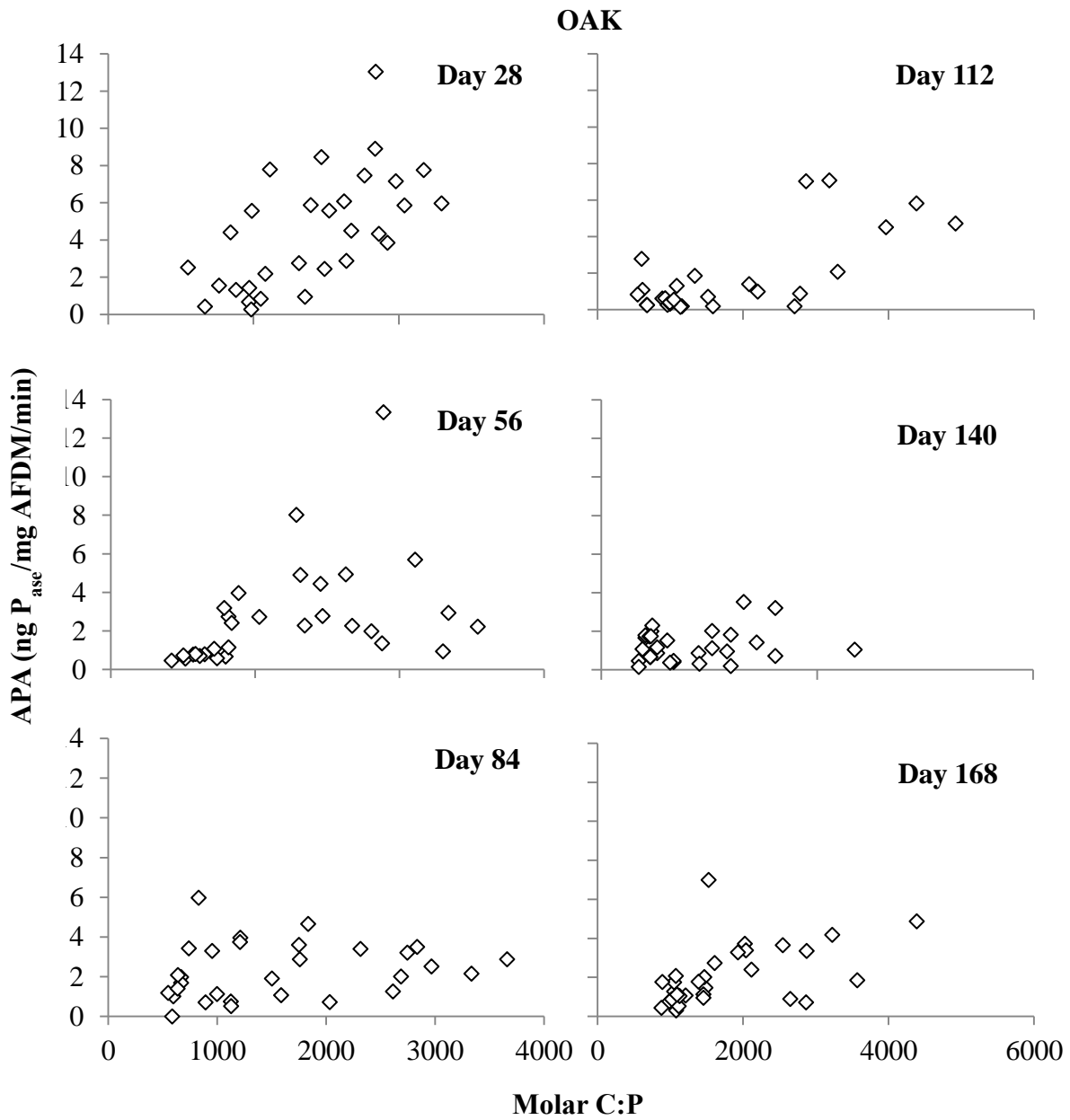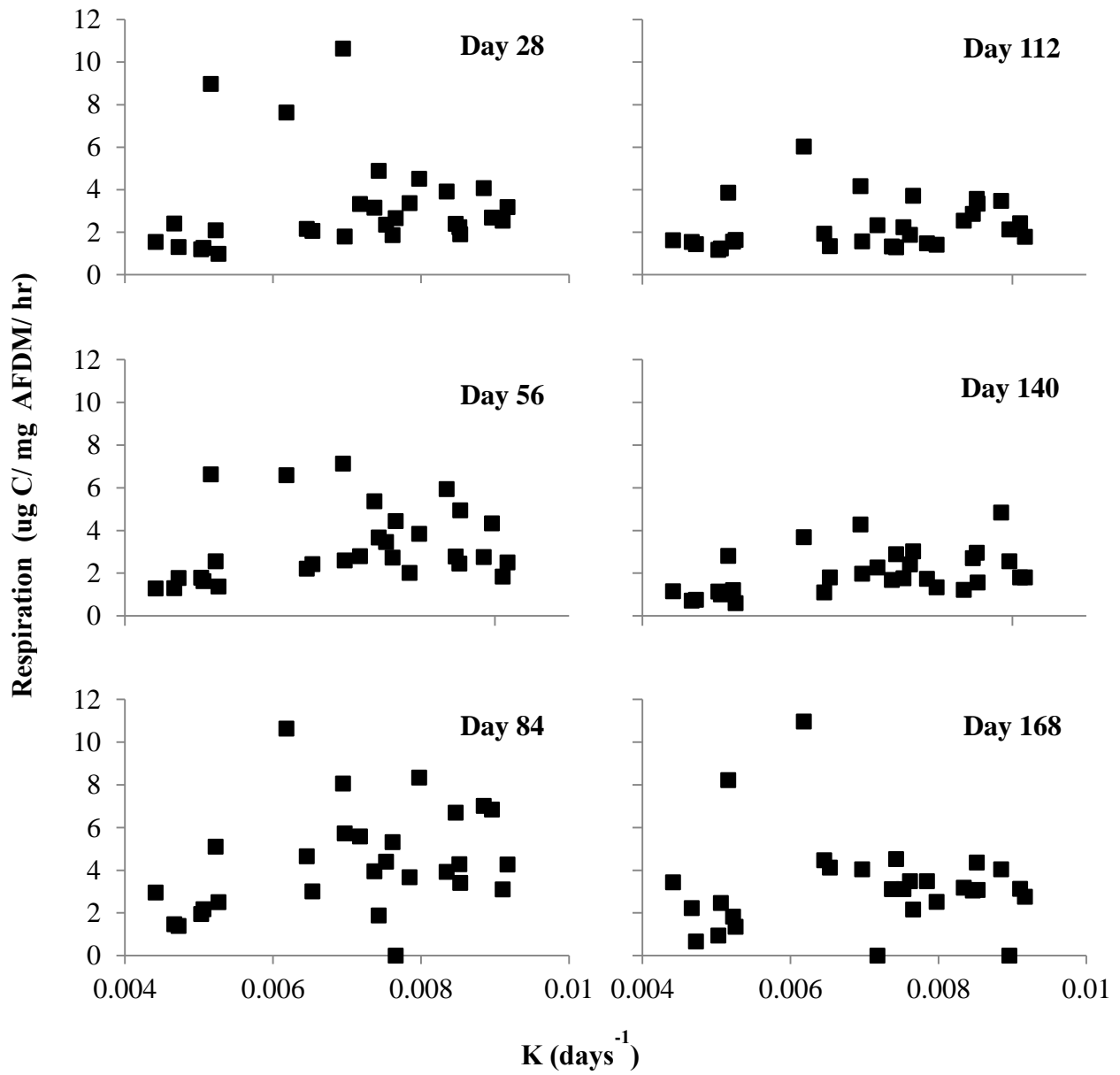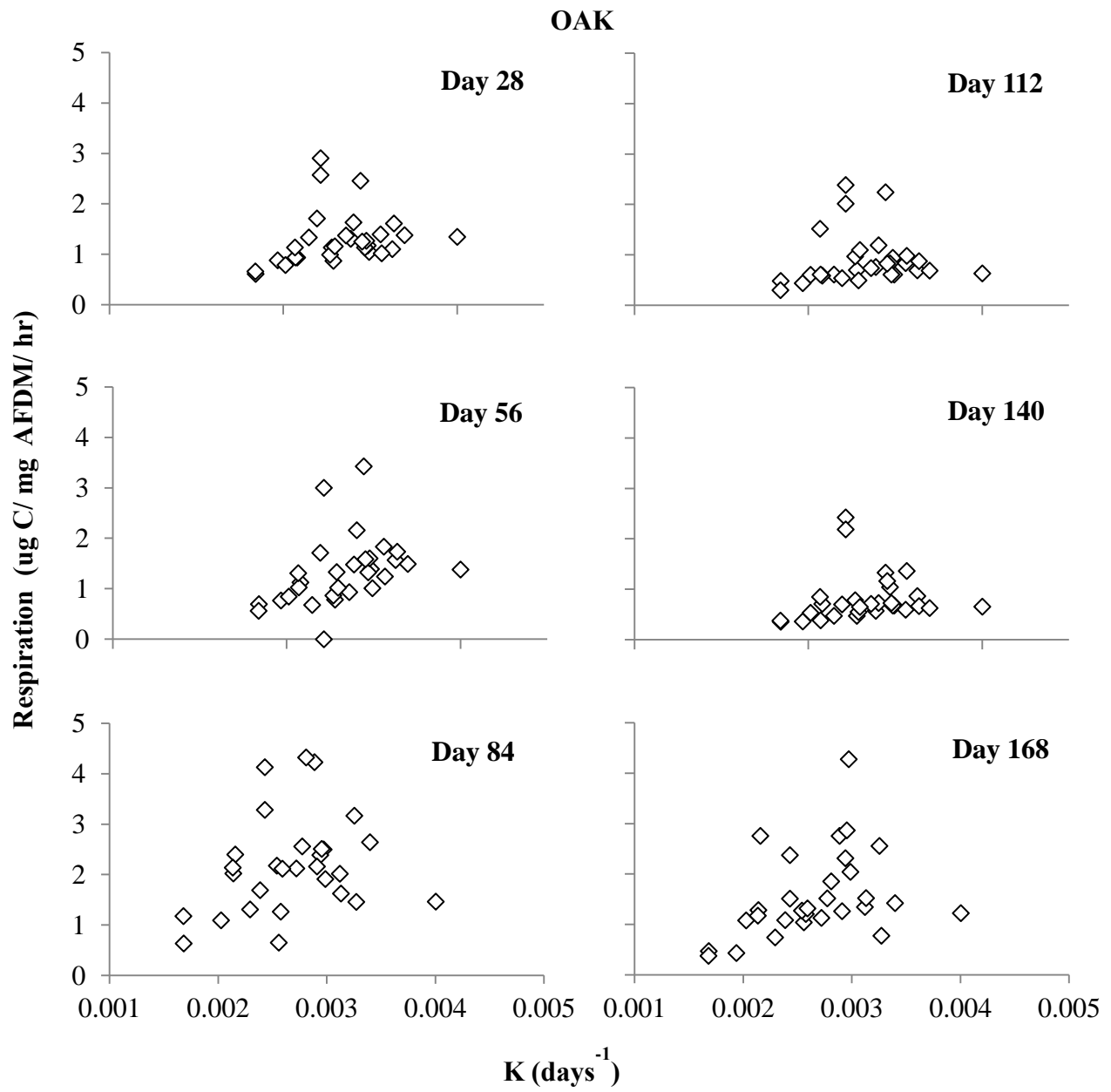
Figure 7

IV.   CONCLUSION

The objective of this study was to quantify the effects of nutrient enrichment on detrital stoichiometry and microbial processes for two leaf types across a wide DIP gradient. Many studies have shown the effects of nutrient enrichment on heterotrophic processes such as increased nutrient content of detrital resources, increased microbial decomposition of detritus, and shifts in community structure and decreases in biodiversity, but few studies have looked at relationships between stoichiometry and microbial processes across such a wide nutrient gradient and long time frame. Due to this large gradient, our study allowed the development of a predictive model to link water DIP concentrations to leaf C:P and decomposition rates. Further, because I measured stoichiometry, decomposition rates, APA, and respiration rates in conjunction with each other, relationships between the variables and along the nutrient gradient could be explored.

Results of the study showed that C:P declined and decomposition rates increased in a saturating relationship along the DIP gradient. Carbon:P values saturated at DIP concentrations of less than 25 µg/L for both litter types, while decomposition rates saturated at approximately 80 µg/L. These concentrations point to values at which increasing DIP enrichment did not elicit further response. Further, while saturation occurred at similar DIP concentrations for maple and oak litter, absolute C:P and decomposition rate saturation points were higher for maple litter compared to oak litter. Differences in the response of stoichiometry and decomposition measurements across the DIP-gradient and differences in the responses of litter type could be important when considering the effects of nutrient enrichment on detrital quality and quantity and subsequent effects on higher trophic levels

Analysis of APA and respiration rates showed no consistent trends through time or across the DIP gradient. Positive correlations were also found between respiration rates and decomposition; however these relationships did not explain much variation in decomposition rate. Again, leaf effects were present for APA and respiration measures, with rates on maple litter being consistently higher than rates on leaf litter. None of these data suggest the use of APA and/or respiration as instantaneous surrogate measures for decomposition in streams.

While decomposition and C:P showed predictive relationships across the DIP gradient that demonstrate patterns of P-limitation in streams, subsequent findings did not support the use of APA and respiration as instantaneous measures of limitation of decomposition in streams. These relationships should be more fully explored to better understand the complex dynamics of microbial limitation and the response to nutrient enrichment. The cumulative results demonstrate that litter type and DIP concentration are both important factors affecting litter quality and quantity in detrital systems and should be considered in nutrient and riparian management.

One caveat to these conclusions is that maple litter and oak litter measurements were treated as statistically independent in order to make comparisons based on litter type. Because the maple and oak litter were incubated together in each mesocosm (though in separate bags) they are not truly independent, and it could be argued that relationships between them are inappropriate to address using my design. Consistent findings of litter type effects, however, suggest that my study findings were not an artifact of the design.