Author's Comments

> Ref. #1: Recent transcriptomic work has shown that each of these different states can be quite complex with somewhere on the order of 200-600 proteins being up or down-regulated based on the nutritional state of the microbes. It seems that only looking at a couple of those proteins is likely to lead to idiosyncratic conclusions.

***We understand the concerns of the reviewer and the advantages offered by transcriptomic approaches. However, enzyme-based modeling of microbial nutrient limitation is a well-recognized approach that has its foundations in a large body of primary literature which has investigated microbial enzyme activities in relation to nutrient dynamics and environmental factors since the early 1970s.

It is of course correct that there are many classes of enzymes involved in the microbial conversion of OM into nutrients (Ljungdahl and Eriksson 1985; Kirk and Farrell 1987; Sinsabaugh 2005; Sinsabaugh et al. 2010), and further, that the expression of the entire suite of enzymes is dependent on nutritional state. However, while many enzymes are active in a general lake ecosystem, only a few will have relatively high activities (Sinsabaugh and Foreman 2001). The four enzymes selected for this study have been repeatedly utilized in terrestrial and aquatic studies alike, slightly varying in combination according to the nature of the research being conducted and the systems of interest (e.g. Sinsabaugh et al. 2010; Mineau et al. 2013; Moorhead et al. 2013; Hill et al. 2014; Parr et al. 2015); this is due to the fact that these enzymes represent the catalysts for *terminal* reactions in which organic matter (OM) is converted into monomer nutrients, as stated in this study (Sinsabaugh et al. 2008; 2010). Important to the extracellular enzyme activity (EEA) assay method is understanding that the bulk of enzymatic pathways devoted to OM degradation converge into those of hydrolytic, terminal catalysts (Allison et al. 2007; Moorhead et al. 2013). BG, NAG, LAP, and AP-mediated breakdown generate low-molecular mass compounds that are readily bioavailable. Together, the activities of these indicator enzymes represent the final steps of OM degradation and are therefore proxies of the total amount of microbial enzyme activity devoted to C, N, or P acquisition (Moorhead et al. 2013).

Significant research has been conducted that shows the inverse correspondence of nutrient availability to specific enzyme activities, for example AP (Wetzel 1981; Chrost & Overbeck 1987; Chrost 1991; Olander & Vitousek 2000; Sinsabaugh et al. 2008; Hill et al. 2010a; b). N-acquiring enzyme activities in relation to N availability is more complicated, but evidence suggests that inorganic N depresses hydrolytic N-acquiring enzyme production (Olander & Vitousek 2000; Stursova et al. 2006) and organic N subsidization induces their production (Sinsabaugh et al. 1997; Weintraub and Schimel 2005; Allison 2007). The development of enzyme studies over the past few decades have established links between environmental nutrient availability and relative enzyme activities, linking ecological stoichiometric and metabolic theories (Sinsabaugh et al. 2009; Sinsabaugh & Shah 2012; Moorhead et al. 2013), leading to models that allow for the prediction of microbial nutrient acquisition efforts and limitation patterns. In support of these models, we note that Referee 2 indicated that we "employed a proper and well-established method for the measurement of enzyme and data interpretation". In short, there is robust evidence that precedes this study to suggest that enzymes are accurate and sensitive indicators of microbial nutrient demand, acquisition efforts, and limitation, and we have cited these studies throughout the manuscript in support of this.

Ref #1: …I think it is trivial to draw a line at a 45 degree angle and suggest that anything above the line is P-limited and anything below the line is N-limited. This kind of information just has not been substantiated enough to say whether that line should be 45 degrees, 48 degrees or 10 degrees.

***In specific response to this comment, we cite Sinsabaugh et al. 2008 & 2009. In these global-scale meta-analyses, Sinsabaugh et al. demonstrate that soil and freshwater sediment EEAs for C-, N-, and P-acquiring enzymes (BG, NAG+LAP, and AP, respectively) converge upon 1:1:1. This suggests that while microbes can allocate portions of their finite resources and energy to nutrient acquisition in response to environmental nutrient scarcity, this plasticity is constrained when considered as a global average. The 1:1:1 ratio is indicative of similar rates of C, N, and P supply across global ecosystems on average, and microbial nutrient limitation by any of these nutrients can occur locally (Sinsabaugh et al. 2009).

The 45° line is therefore not arbitrary, but empirically founded on global evidence that C:N and C:P enzyme activity ratios will be equal (the slope of the C:N vs. C:P line will be 1, or the angle 45°) within an "average" ecosystem systems that is not limited by C, N, or P.

We address this by making the following change within our manuscript:

p. 8, line 14: "Across ecosystems, nutrient acquisition effort as measured by BG, NAG+LAP, and AP is typically close to 1:1:1 *based on global empirical evidence and* following stoichiometric and metabolic theories (Sinsabaugh et al. 2008, *2009*)."

The derivation of the 45° line from these enzyme activity relationships is further explained in the methods that follow this addition.

Benjamin Burpee 12/2/2015 9:46 AM
Comment [1]: Updated author's response

Ref. #1: I also have concerns about the execution of the study in particular, although I know it is problematic doing research in remote places, the authors froze the samples for transport from the sites back to the USA or NZ where analyses were conducted. No mention is made of any controls or quality control to determine if freezing had any effect on enzyme activity. This would be particularly problematic if it affected some enzymes differently than others which would certainly affect the conclusions of the study. It would also be useful to know that each of the enzymes was measured at the Vmax, providing a solid quantitative measure of enzyme activity.

Ref. #2: I can see that collection and storage of samples were highly difficult due to the location of study sites, but freeze-and-thawing affects and often substantially interfere with enzyme

activities. The authors should explain possible problems or a source of error due to the sample treatment.

***Storage of enzyme samples was indeed dictated by the remote location of our study sites and the transport options available to us. There is no current consensus on the effects of freezing on measures of enzyme activity. For example, in a review of enzyme methodology German et al. (2011) found no consistent effects of freezing. Some studies have found no significant difference of enzyme activities from soil samples stored refrigerated or frozen (Lee et al. 2007; DeForest 2009). Wallenius et al. (2010) propose that freezing has only minor effects on enzyme activity samples, especially within uniform sample types. In streams, the effects of freezing can be variable (Smucker et al. 2009) although explicit tests are restricted to a few locations. We note that there is precedence in other investigations that have successfully used frozen water samples for EEA analysis (e.g. Simon et al. 2009; Clinton et al. 2010; Freimann et al. 2013; Parr et al. 2015). Since there was no way to analyze fresh samples from Greenland, control samples were not available. To address this issue, we will add the following caveat into the methods section:

"Due to the remote location of the lakes samples from June were stored frozen (−20 ◦C) for 60 days and samples from July were refrigerated for 30 days and then frozen for 30 days before analysis. *Though the analysis of fresh samples is considered preferable due to the uncertainty of whether freezing introduces bias into results, it is common for freshwater EEA studies to freeze samples owing to logistical constraints (e.g. Simon et al. 2009; Clinton et al. 2010; Freimann et al. 2013; Parr et al. 2015). We are assuming that if freezing had any effect it was similar across systems.* EEA samples were thawed, processed and analyzed…"

It is important that each of the enzymes is measured at V_{max}, which was why we experimentally determined the saturating concentration of substrates prior to the study. To make this clearer, we will add the following amendment to the methods section:

"Pilot assays were used to ensure substrate concentrations saturated enzyme kinetics*, such that kinetic rates were equal to* V_{max}, and readings were made during linear increases in fluorescence. Throughout the analysis…"

Ref. #1: Another concern here is that the authors refer to the organisms producing the exoenzymes as 'bacteria', but I doubt they looked to see if there were also archaea and/or eukaryotes in their samples. If so, they should mention it.

***This is an excellent point. Though we did not determine archaeal, algal, or even bacterial abundance, we are cognizant of the fact that extracellular enzymes in aquatic environments are produced by phyto- and bacterioplankton alike. This is why both bacteria and phytoplankton studies were considered throughout the paper. Though we were careful with our wording, we inadvertently specified 'bacteria' in a few places. We refer now exclusively to "microbes" to encompass all possible taxonomic groups that could be involved in enzyme production.

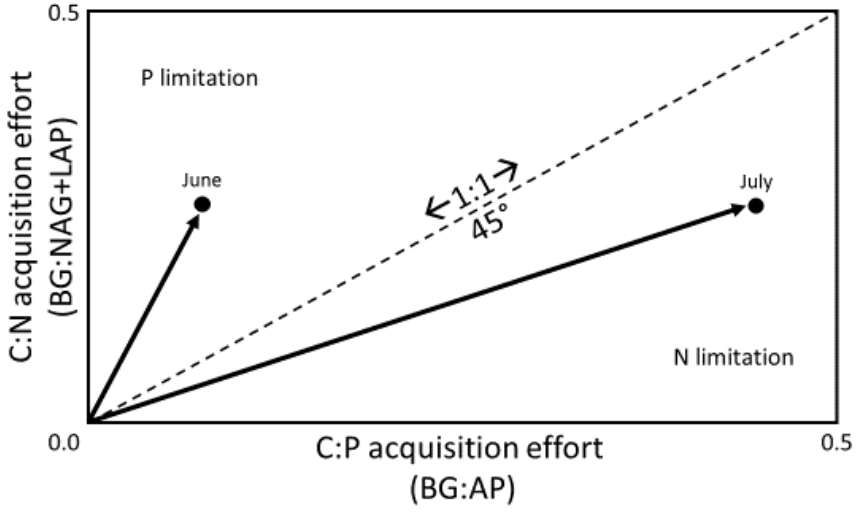
Ref. #1: Presentation quality (rating: 2) The authors use an approach adopted from Moorhead et al. 2013 to determine nutrient limitation from enzyme data whereby a vector length and angle are calculated. I had to look at several papers before I found a decent description with the mathematics of this approach (Hill et al. 2014). The description should also be included in the present manuscript because most readers will not be very familiar with it.

***We cite Hill et al. 2014 when explaining this approach, and will insert the following into line 18 of p. 11870 to make the method more intuitive to readers:

"Figure 2 displays hypothetical data from a lake in June and July plotted onto a vector plot with the 1:1 line drawn in dashes. The vectors from which angles are calculated are shown as arrows from the origin to the individual data points. In June, the vector angle is positive with respect to the 1:1 line (> 45 ^{o}) indicating P limitation in this lake. However, in July nutrient limitation shifts from P to N, as indicated by the negative angle with respect to the 1:1 line (< 45 ^{o})."

And we will add the following figure and caption:

Figure 2. An example of vector plot analysis for a hypothetical lake sampled in June and July. The 1:1 line is drawn in dashes and separates zones of P imitation (above) from N limitation (below). Vectors for each data point are drawn in arrows. Their angles indicate microbial nutrient limitation, such that the positive angle value with respect to the 1:1 line in June indicates P limitation, while the negative one in July indicates a shift to N limitation. The lengths of the vectors are also indicative of microbial C acquisition efforts, which in this example is greater in July than in June.



Figures will be renumbered in the final submission to make this addition consistent.

> Ref. #1: It is also complicated by the fact that several of the figure axes and captions in the paper seem to be mis-labeled or not labeled at al. Units in Fig. 3 are not given and the ratios in that figure for BG:NAG+LAP are on the order of 10-60. But then in Fig. 4, the axis for BG:NAG+LAP is in the range of 0-0.8. The caption says that what is plotted are the vector angles, i.e., not the activity, but in Table 2 the vector angle ranges are around -10 to +45. So it is really not clear what is being plotted in Fig. 4. Figure 4 also seems like a more convoluted plot than it needs to be. If BG is in the numerator for each axis, it cancels itself out and essentially they are plotting NAG+LAP against AP and therefore should be labeled that way. Figs. 5 and 6 also need units to be labeled.

***Units in Figure 3 are not given due to the fact that the quantities being shown are ratios, and therefore without units. You may likely be referring to Figure 2, where the units are not given on the axis labels, but are instead reported in the figure caption in order to save space. This has been the convention in other enzyme papers, and we use it here. For Figures 5 and 6, the variables are ratios or natural log (ln)-transformed quantities are therefore without units. You picked up on a discrepancy in our data that was due to a calculation error—the BG:NAG+LAP ratios reported in Figure 4 are accurate, whereas those in Figure 3 are erroneous. This mistake will be fixed for the final submission, but importantly, it does not change our significant findings and overall conclusions of the paper.

Figure 4 is indeed complex, and we heed your suggestions. We address this by changing the caption:

"Figure 4. Scatterplot of microbial enzyme ratios (BG : NAG + LAP vs. BG : AP) about the 1:1 line. Included is C:P and C:N acquisition data of lake epilimnia (circles) and hypolimnia (triangles) from June (gray) to July (black). Dotted line indicates 1:1 (45 °) line. Vector angles (indicative of nutrient limitation) are calculated from these plotted data points, as deviation from the 1:1 line."

We disagree that BG should be cancelled out, though it is the numerator in both variables. If we were seeking regression between the two variables, then BG would cause covariation. However, this is not our intention. We are instead interested in the angles generated by the data points plotted using the two ratios *and* we were interested in determining microbial C acquisition efforts by vector lengths, despite the fact that C acquisition did not show important trends in this study. Regardless, obtaining and analyzing C-acquisition data would not have been possible if BG had been factored out.

> Ref. #1: More specific comments: p. 11873: Why did they use DIN: TP as an index of nutrient limitation? A more appropriate comparison would be DIN:DIP or TN:TP. p. 11874 line 15: I don't think you can necessarily infer that the DOM supply was poor in P from this relationship. There can be (and likely are) other sources of N and P other than DOM. Also, it is the supply relative to the requirements of the organisms that would determine this relationship.
>
> Ref. #2: High correlation between DOM and TN and absence of such relationship between DOM and TP do not necessarily indicate the difference in availability between N and P. Rather,

different chemical properties of organic N (which is mostly directly bonded to C) and organic P (mostly tied as an ester bond) could be the reason for that.

***We believe that DIN:TP is a more accurate account of nutrient limitation than DIN:DIP or TN:TP based on the work of Bergstrom (2010) in which DIN:TP serves as a better indicator of phytoplankton nutrient limitation than TN:TP in oligotrophic lakes. Though our enzyme samples include activities from both algal and bacterial organisms, presumably with different stoichiometric requirements, the DIN:TP ratio seemed most appropriate, especially since DIP (PO_4^{3-}) was frequently below detection limits. Further, DIP is typically not used because P can cycle extremely rapidly and so the total pool of P is considered a better index of P availability. This argument does not hold for N, as it does not cycle as quickly. Also, as stated in the manuscript, TN and DOC positively covaried, making it difficult to separate their effects, and importantly, the size of the TN pool in high DOM lakes does not necessarily correlate to bioavailability of N.

To avoid suggesting that DOM is a poor P supply on p.11874, we will change the sentence to the following: “Collectively, these enzyme and water chemistry data suggest that the DOM in these lakes may provide a readily available source of N, while higher DOM concentrations are associated with enzyme-mediated microbial P acquisition.”

Ref. #2: One reservation for the paper is about C- mineralizing enzyme. Most of DOM delivered to lakes could be composed of highly recalcitrant carbon for which beta-glucosidase may not be a representative enzyme. Decomposition of phenolic or humic materials is known to be harnessed by oxidase activity (e.g., phenol oxidase or laccase), which in turn may limit the activities of other hydrolases (see, Freeman et al., 2001). Enzymes involved in mineralization of recalcitrant carbon should be discussed somewhere in the manuscript.

The authors note the importance of oxidative enzymes in the degradation of recalcitrant forms of OM. BG is a more commonly utilized enzyme in aquatic literature, as it is assumed to broadly represent C acquisition activity. However, an enzyme such as phenol oxidase could have provided insight into degradation rate of terrestrially-derived C. We will address this in the manuscript discussion on page 11876:

“Bacterial community structure has been shown to change in correspondence with DOM quality in arctic lakes, as some bacteria prefer more labile compounds while other species are adapted to utilizing recalcitrant forms (Crump et al., 2003). *In this study, the seasonal source and quality of the DOM pool might have been inferred by the inclusion of oxidative enzymes, such as phenol oxidase or peroxidase, which are responsible for degrading terrestrially-derived compounds such as phenols and aromatics, respectively (Sinsabaugh et al. 2008). Though BG is assumed to broadly represent C acquisition activity, oxidative enzyme activity may be an important metric in future studies*.”

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List of Manuscript changes:

1. p. 2, line 1: “bacteria” changed to “microbes”

2. p. 7, line 18, added: “Though the analysis of fresh samples is considered preferable due to the uncertainty of whether freezing introduces bias into results, it is common for freshwater EEA studies to freeze samples owing to logistical constraints (e.g. Simon et al. 2009; Clinton et al. 2010; Freimann et al. 2013; Parr et al. 2015). We are assuming that if freezing had any effect it was similar across systems.”

3. p.8, line 8, added: “…such that kinetic rates were equal to Vmax,…”

4. p. 8, line 14, added: “…based on global empirical evidence and”, and on line 15 added Sinsabaugh et al. 2009 citation.

5. p. 8, line 18, changed citation format.

6. p. 9, line 1, added: “…(45°)…”

7. p. 9, line 2, added: “Figure 2 displays hypothetical data from a lake in June and July plotted onto a vector plot with the 1:1 line drawn in dashes. The vectors from which angles are calculated are shown as arrows from the origin to the individual data points. In June, the vector angle is positive with respect to the 1:1 line (> 45°) indicating P limitation in this lake. However, in July nutrient limitation shifts from P to N, as indicated by the negative angle with respect to the 1:1 line (< 45°). We quantified vector angles…”

8. p. 10, line 21: sentence adjusted to reflect corrected data.

9. p. 11, lines 2 & 3: sentences adjusted to reflect corrected data.

10. p. 11, line 10, added: “...greater in…”

11. p. 12, line 13, “heterotrophic bacteria” changed to “bacterioplankton”

12. p. 13, line 7, sentence changed per reviewer’s suggestion: “Collectively, these enzyme and water chemistry data suggest that the DOM in these lakes may provide a readily available source of N, while higher DOM concentrations are associated with enzyme-mediated microbial P acquisition.”

13. p. 15, line 10, added: “In this study, the seasonal source and quality of the DOM pool might have been inferred by the inclusion of oxidative enzymes, such as phenol oxidase or peroxidase, which are responsible for degrading terrestrially-derived compounds such as phenols and aromatics, respectively (Sinsabaugh et al. 2008). Though BG is assumed to broadly represent C acquisition activity, oxidative enzyme activity may be an important metric in future studies.”

14. p. 15, line 20: “bacteria” changed to “microbes”

15. p. 15, line 23, deleted: “Lastly, the strength of DOM as a determining factor of nutrient dynamics increased later in the summer.”

16. p. 17, line 15, added citation: Clinton, S. M., Edwards R. T., and Findlay S. E. G.: Exoenzyme activities as indicators of dissolved organic matter composition in the hyporheic zone of a floodplain river, Freshwater Biol., 55, 1603-1615, 2010.

17. p. 18, line 13, added citation: Freimann, R., Bürgmann, H., Findlay, S. E., and Robinson, C. T.: Response of lotic microbial communities to altered water source and nutritional state in a glaciated alpine floodplain, Limnol. Oceanogr., 58, 951-965, 2013.

18. p. 19, line 20: citation adjusted.

19. p. 21, line 20: citation adjusted.

20. p. 22, line 1: citation adjusted.

21. p. 22, line 14, added citation: Simon, K. S., Simon, M. A., and Benfield, E. F. Variation in ecosystem function in Appalachian streams along an acidity gradient, Ecol. Appl., 19, 1147-1160, 2009.

22. p. 23, lines 5 & 7: citations adjusted.

23. p. 27, line 3, added: “Figure 2. An example of vector plot analysis for a hypothetical lake sampled in June and July. The 1:1 line is drawn in dashes and separates zones of P imitation (above) from N limitation (below). Vectors for each data point are drawn in arrows. Their angles indicate microbial nutrient limitation, such that the positive angle value with respect to the 1:1 line in June indicates P limitation, while the negative one in July indicates a shift to N limitation. The lengths of the vectors are also indicative of microbial C acquisition efforts, which in this example is greater in July than in June.”

24. p. 27, line 15, adjusted figure legend: “Figure 5. Scatterplot of microbial enzyme ratios (BG : NAG + LAP vs. BG : AP) about the 1:1 line. Included is C:P and C:N acquisition data of lake epilimnia (circles) and hypolimnia (triangles) from June (gray) to July (black). Dotted line indicates 1:1 (45 °) line. Vector angles (indicative of nutrient limitation) are calculated from these plotted data points, as deviation from the 1:1 line.”

25. Throughout text: Figure numbers have been adjusted accordingly for the addition of Figure 2.

Microbial nutrient limitation in arctic lakes in a permafrost landscape of southwest Greenland

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Abstract

Permafrost is degrading across regions of the Arctic, which can lead to increases in nutrient concentrations in surface freshwaters. The oligotrophic state of many arctic lakes suggests that enhanced nutrient inputs may have important effects on these systems, but little is known about microbial nutrient limitation patterns in these lakes. We investigated microbial extracellular enzyme activities (EEAs) to infer seasonal nutrient dynamics and limitation across 24 lakes in southwest Greenland during summer (June and July). From early to late summer, enzyme activities that indicate microbial carbon (C), nitrogen (N), and phosphorus (P) demand increased in both the epilimnia and hypolimnia by 74% on average. Microbial investment in P acquisition

was generally higher than that for N. Interactions among EEAs indicated that microbes were primarily P limited. Dissolved organic matter (DOM, measured as dissolved organic carbon) was strongly and positively correlated with microbial P demand (R^2 = 0.84 in July), while there were no relationships between DOM and microbial N demand. Microbial P limitation in June epilimnia (R^2 = 0.67) and July hypolimnia (R^2 = 0.57) increased with DOM concentration. The consistency of microbial P limitation from June to July was related to the amount of DOM present, with some low DOM lakes becoming N-limited in July. Our results suggest that future changes in P or DOM inputs to these lakes are likely to alter microbial nutrient limitation patterns.

Benjamin Burpee 11/27/2015 12:24 PM
Deleted: bacteria

1 Introduction

Permafrost degradation is one of the most prominent responses of arctic landscapes to accelerated warming. Many factors can influence the thaw rate of permafrost (Zhang et al. 2005; Jorgenson et al. 2010), but permafrost thaw is very sensitive to even small changes in air and ground temperatures (Hinkel and Nelson 2003; Zhang et al. 2005; Romanovsky et al. 2007; White et al. 2007). Permafrost is expected to continue to degrade in response to climate warming (Jorgensen et al. 2001; Lawrence and Slater 2005; Jorgensen et al. 2006; Frey et al. 2009), with some models predicting that in areas of continuous permafrost, near-surface permafrost extent will decrease by 26-90% (Lawrence and Slater 2005; Anisimov and Reneva 2006) and soil active layer depth will deepen by 30-100% (Stendel and Christensen 2002; Zhang et al. 2008a) over the next century. Such changes are likely to alter biogeochemical cycling in soils and the aquatic systems that receive material from soils.

The soil active layer controls much of the tundra landscape's hydrologic and biogeochemical activity (Hinzman et al. 1991; Waelbroeck et al. 1997; Zhang et al. 2005; Schuur et al. 2008; Frey et al. 2009), which in turn affects groundwater and nutrient inputs to arctic aquatic ecosystems (Hobbie et al. 1999; Zhang et al. 2005; White et al. 2007). Degradation of permafrost typically increases phosphorus (P) export to surface waters (Hobbie et al. 1999, Frey and McClelland 2009) while changes in inorganic nitrogen (N) and dissolved organic carbon (DOC) are less consistent. For example, with permafrost thaw, watershed DOC export in the Yukon, Alaska, and Central Siberia decreased (Carey 2003; Kawahigashi et al. 2004; Striegl et al. 2005; McClelland et al. 2007) whereas it increased in West Siberia (Frey and Smith 2005).

These water chemistry changes are important for the ecology of arctic lakes because they alter nutrient and energy availability to phytoplankton and heterotrophic microbes. Hobbie et al. (1999) demonstrated that permafrost thaw in Northern Alaska contributed 30% of inflowing phosphate and nitrate into Toolik Lake. Long-term experimental manipulation of another lake at that study site demonstrated that sustained P inputs increased primary production and increased phytoplankton biomass (Hobbie et al. 1999). It was therefore speculated that P inputs from permafrost degradation would increase lake eutrophication. However, another experimental study on Alaskan arctic lakes indicated that N subsidies may be more important than P in stimulating phytoplankton productivity (Levine and Whalen 2001). Despite these few studies, the nature and magnitude of permafrost degradation effects on arctic lakes remain largely uncharacterized and largely focused on phytoplankton production.

Heterotrophic bacteria are key to aquatic biogeochemical reactions and transformations and they should be susceptible to changes in DOC and nutrient input to lakes in arctic systems (Cotner and Biddanda 2002, Villar-Argaiz et al. 2002, Crump et al. 2003). Due to microbial

mineralization of C, arctic lakes can release significant amounts of greenhouse gases, such as CO_2 and CH_4 (Kling et al. 1992). In arctic lakes, the source of DOM can shift seasonally from the landscape (allochthonous DOM) to in-lake production (autochthonous) (Whalen and Cornwell 1985). This can shift microbial community structure and production rates (Crump et al. 2003). In aquatic systems receiving nutrient subsidies, nutrient limitation of bacteria should relax. This would increase the rate by which heterotrophic bacteria consume organic matter for growth and respiration, resulting in increased CO_2 production in oxygenic environments, or CH_4 in anoxic ones (del Giorgio and Cole 1998; Smith and Prairie 2004). This has important implications for arctic lakes that may receive nutrient subsidies through permafrost degradation. For instance, microbial growth increased in lake and pond waters of the high Canadian Arctic that received experimental P subsidies, indicating microbial P limitation (Graneli et al. 2004). Alternatively, in lakes that receive fewer hydrological inputs of nutrients and DOM due to increased soil active layer depth and catchment microbial activity, the size of DOM and nutrient pools would decrease. Such a decrease would initiate a simplification of lake microbial food web structure (Hobbie et al. 2000). Further investigating heterotrophic microbial activity in arctic lakes at present is important to understanding future thaw-driven changes in nutrient inputs.

One way to assess the nutrient demands of microbial communities is to measure activities of extracellular enzymes (EEA), used by microbes to cleave complex organic molecules into smaller compounds that can be assimilated. Relative activities of enzymes associated with C, N and P acquisition can be used to infer nutrient limitation following resource allocation models (Sinsabaugh et al. 2008). Enzymes of interest in EEA studies commonly include β-1,4-glucosidase (BG) that degrades cellulose and β-1,4-glucans to glucose for C acquisition (Ljungdahl and Eriksson 1985; Sinsabaugh et al. 2008), β-N-acetylglucosaminidase (NAG) and

leucine aminopeptidase (LAP) which acquire N from chitin and polypeptides, respectively, (Sinsabaugh and Foreman 2001; Sinsabaugh et al. 2008), and phosphatase (AP) which degrades phosphomonoesters to obtain P (Turner et al. 2002; Sinsabaugh et al. 2008). These enzymes are catalysts for terminal reactions in which organic matter is converted to monomer nutrients (Sinsabaugh et al. 2008). As such, their activity reflects total microbial demand for C (via BG), N (via NAG+LAP), and P (via AP). Recent work has established the use of EEAs as a method to infer microbial nutrient limitation (Sinsabaugh et al. 2008; Moorhead et al. 2013, Hill et al. 2014), making EEA assays an extremely valuable tool for evaluating changing nutrient concentrations in aquatic ecosystems.

We examined microbial nutrient limitation patterns, via EEA analysis, and water chemistry in lakes spanning a range of nutrient availability in the continuous permafrost landscape of southwest Greenland. While the Alaskan, Siberian, and Canadian Arctic have shown consistent increases in air temperatures and active layer thickening since the mid-1970's (Blunden and Arndt 2014), recent and abrupt (>10°C) warming in western Greenland (Hanna et al. 2012) coincides with deepening permafrost active layer only since the mid 1990's (Christiansen et al. 2010). As a result, these relatively recent changes in Greenland provide a unique situation in which we could examine patterns in microbe-nutrient relationships in a landscape with relatively low permafrost loss, providing a baseline from which to gauge future change. We measured EEA during the summer of 2013 in 24 lakes situated around Kangerlussuaq, southwest Greenland. We hypothesized that most lakes would be P limited based on previous findings with phytoplankton experiments in this area (Brutemark et al. 2006), but that patterns in microbial enzyme allocation toward C, N and P would track variation in lakewater DOC, dissolved inorganic N (DIN), and total P (TP) availability.

2 Methods

2.1 Study site

The region around Kangerlussuaq, Greenland (67°00' N, 50°41' W, Figure 1) contains more than 20,000 lakes (Anderson et al. 2001) and is underlain by continuous permafrost estimated to be 300 m thick (Nielson 2010; Harper et al. 2011). The climate of this region is low Arctic continental with a mean summer temperature of 10.2° C. In Western Greenland, annual air temperature has increased by 3°C and annual melting degree days by 100% when comparing 2007-2012 to 1979-2000 (Mayewski et al. 2014). The region is semi-arid, receiving approximately 150 mm of precipitation per year, and even less at the ice sheet margin. Lakes in this study ranged in surface area from 0.02 to 0.8 km^2, and in maximum depth from 9 to 36 m. Most of these lakes are oligotrophic, with low nutrient concentrations characteristic of lakes in this region (Anderson et al. 2001, Perren et al. 2009). Lakes were first sampled in June shortly following ice-off. At that time, about half of the study lakes were weakly stratified. For those that were not, the "hypolimnion" sample depth was the estimated limit of the euphotic zone determined as twice the measured Secchi depth. Lakes were sampled again in July, during the period of stable thermal stratification for all lakes.

2.2 Environmental parameters

Physical and chemical variables of the lakes were measured to determine their relationship to microbial EEAs within the epilimnia of the study lakes. Temperature and pH were measured at the point of greatest lake depth using a submersible HydroLab Datasonde 5a. Epilimnetic and hypolimnetic water samples were collected with a van Dorn bottle. Water samples for measurement of dissolved nutrients and DOC were filtered through Whatman GF/F

filters that were pre-rinsed with DI water. Samples for total nutrients were unfiltered. All samples were collected into acid-washed bottles and kept refrigerated until analysis. Dissolved inorganic (NH_4^+, NO_3^-, and PO_4^{3-}) and total nutrient (TN and TP) concentrations were analyzed on a Lachat QuickChem 8500 flow injection analyzer. Nitrate was measured with the cadmium reduction method, NH_4^+ with the phenate method, and PO_4^{3-} with the ascorbic acid method (APHA 2000). TN and TP were determined by measurement of NO_3^- and PO_4^{3-} on unfiltered water samples following digestion with persulfate (APHA 2000). Quantification limits on all nutrients were 2 µg L^{-1} except for TN, which was 10 µg L^{-1}. For statistical analyses, nutrient values below the 2 µg L^{-1} quantification limit were replaced with 1. Dissolved inorganic nitrogen: TP (DIN:TP) ratios were calculated, with DIN determined by the addition of NH_4^+ and NO_3^-. The DIN:TP ratio is a useful metric for inferring nutrient limitation, moving from N to P limitation with an increase from 1.5 to 3.4 (Bergstrom 2010). DOC was analyzed with an OI Analytical Aurora 1030D TOC analyzer using wet chemical oxidation.

2.3 EEA analysis

Water samples for EEA analysis were collected in the same way as total nutrient samples (i.e., not filtered). Due to the remote location of the lakes samples from June were stored frozen (-20°C) for 60 days and samples from July were refrigerated for 30 days and then frozen for 30 days before analysis. Though the analysis of fresh samples is considered preferable due to the uncertainty of whether freezing introduces bias into results, it is common for freshwater EEA studies to freeze samples owing to logistical constraints (e.g. Simon et al. 2009; Clinton et al. 2010; Freimann et al. 2013; Parr et al. 2015). We are assuming that if freezing had any effect it was similar across systems. EEA samples were thawed, processed and analyzed with a Thermo Electron Corporation Fluoroskan Ascent FL fluorescence spectrophotometer using fluorescent-

labeled substrates following published methods (Sinsabaugh and Foreman 2001; Findlay et al. 2003). Fluorescent substrates were used to measure activity of BG (4-MUB-β-D-glucoside), NAG (4-MUB-N-acetyl-β-D-glucosaminide), LAP (L-Leucine-7-AMC) and AP (4-MUB-phosphate). Briefly, 200 µL sub-samples from each lake sample were added in triplicate to 96-well assay plates and combined with 50 µL of substrate for a final saturated substrate concentration of 200 µM and assayed at 25°C. Controls for substrate and sample fluorescence and quenching were included. Pilot assays were used to ensure substrate concentrations saturated enzyme kinetics such that kinetic rates were equal to V_{max}, and readings were made during linear increases in fluorescence. Throughout the analysis, steps were taken to standardize and optimize the procedure following the suggestions of German et al. (2011).

2.4 Data analysis

Microbial nutrient limitation was inferred from activity of individual enzymes and from ratios of multiple enzymes. Across ecosystems, nutrient acquisition effort as measured by BG, NAG+LAP, and AP is typically close to 1:1:1 based on global empirical evidence and following stoichiometric and metabolic theories (Sinsabaugh et al. 2008, 2009). Departures from these values are indicative of differential microbial nutrient demand as microbes invest resources in enzymes to acquire limiting nutrients. Degree of C limitation can be inferred from ratios of C to nutrient acquiring enzymes (BG:NAG+LAP and BG:AP; Sinsabaugh et al. 2008, 2009). Further, the stoichiometric ratios BG:NAG+LAP and BG:AP can be considered in concert to gauge degree of microbial N or P limitation (Moorhead et al. 2013; Hill et al. 2014). This can be done by plotting BG:NAG+LAP vs. BG:AP and measuring deviation from the 1:1 line which indicates equal nutrient acquisition effort (Sinsabaugh et al. 2008; Sinsabaugh et al. 2009; Moorhead et al. 2013; Hill et al. 2014). On these plots the distance from the origin to a data point

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forms a vector. Deviation of the vector angle from the 1:1 (45°) line indicates increasing P (higher angles) or N (lower angles) limitation (Moorhead et al. 2013; Hill et al. 2014). Figure 2 displays hypothetical data from a lake in June and July plotted onto a vector plot with the 1:1 line drawn in dashes. The vectors from which angles are calculated are shown as arrows from the origin to the individual data points. In June, the vector angle is positive with respect to the 1:1 line (> 45°) indicating P limitation in this lake. However, in July nutrient limitation shifts from P to N, as indicated by the negative angle with respect to the 1:1 line (< 45°). We quantified vector angles for our samples and display the data as degrees of deviation from the 1:1 line such that positive values indicate P limitation and negative ones indicate N limitation (Figure 5). Lastly, the distance of data points from the origin indicates microbial investment in C acquisition relative to that of N and P, such that C demand is indicated by larger vector lengths (Moorhead et al. 2013, Hill et al. 2014).

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To test whether water quality parameters, nutrient concentrations, or enzyme-related activities differed between months (June vs. July) or lake strata (epilimnia vs. hypolimnia), two-tailed, paired t-tests were used. To determine whether certain factors, such as nutrient concentrations or ratios, were related to enzyme activities, simple least squares linear regression was used with a level of significance of $p = 0.05$. All statistical analyses were completed using R (version 3.1.2).

3 Results

3.1 Water quality parameters

Several physical and chemical parameters varied from June to July in lake epilimnia (Table 1; Supplementary Table 1). Surface water temperatures increased between June and July,

on average from 7.8° to 13.5°C ($p < 0.01$). pH was relatively consistent across lakes (5.8 – 7.7) but on average increased from 6.6 in June to 7.0 in July ($p < 0.01$). DOC ranged tenfold from 4 to 40 mg L^{-1} across study lakes, and slightly increased, on average, over the summer (12.1 to 13.4 mg L^{-1}, $p < 0.01$). DIN ranged from 2 to 25 µg L^{-1}, and increased from June to July (6 to 12 µg L^{-1}, $p < 0.01$), as did TN (467 to 554 µg L^{-1}, $p < 0.01$, range from 178 to 1132 µg L^{-1}). TP ranged from <2 to 12 µg L^{-1} and did not appreciably increase from June to July (4 to 5 µg L^{-1}, $p = 0.68$). PO_4^{3-} was below quantification limits at all times. DIN:TP ranged from 0.17 to 22 and increased from June to July (2.1 to 5.4, $p < 0.01$). TN was tightly related to DOC in both months (June; $R^2 = 0.83$, $p < 0.01$; July $R^2 = 0.77$ $p < 0.01$). There were no relationships between TP and DOC, or DIN:TP and DOC.

3.2 Seasonal and spatial patterns in enzyme activities

Activity of all enzymes increased from June to July in both the epilimnia (p values ≤ 0.03) and hypolimnia (p values ≤ 0.02; Figure 3). Averaged between lake strata, BG activity increased 73%, NAG+LAP 79%, and AP 70% from June to July. Averaged across lakes, absolute activities of single enzymes differed between epilimnetic and hypolimnetic samples. For BG, epilimnetic activities were 1.7x higher than those of hypolimnia in June ($p = 0.01$) and 1.3x higher in July ($p = 0.42$). For NAG+LAP, epilimnetic activities were 1.1x and 1.3x higher than those of hypolimnia in June and July ($p < 0.01$ and $p = 0.01$, respectively). There were no differences in AP activities between strata in either month (p values > 0.05).

Ratios of C to nutrient-acquiring enzyme activity varied by lake strata and time (Figure 4). Hypolimnetic BG:AP was consistently lower than epilimnetic BG:AP, though the difference was not significant in July (June $p = 0.01$, July $p = 0.09$). In June, epilimnetic BG:NAG+LAP was greater than that of the hypolimnia ($p = 0.03$), but in July there was no difference ($p = 0.72$).

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There were no seasonal differences in BG:AP or BG:NAG+LAP within the same stratum (p values < 0.05). BG:NAG+LAP was greater than BG:AP in epilimnia and hypolimnia in June (p values < 0.01) but not in July (p values > 0.05). These data suggest microbes were investing more in P and less in N acquisition across lakes in June but not July.

Vector angles were mostly positive indicating consistent microbial P limitation across all lakes in both time periods (Figure 5). In the epilimnia, the angle magnitude was about 3 times lower in July than in June suggesting relaxed P limitation later in the year ($p < 0.01$, Table 2). This was less obvious in the hypolimnia where June and July data were much more similar ($p = 0.04$). Angles in the hypolimnia were 1.3x greater than those of the epilimnia in June and 3.6x greater in July (p values < 0.01). On the vector plots, distance of data points from the origin did not change between June and July within the same strata (Table 2). This indicates that microbial investment in C acquisition did not appreciably vary with respect to N- and P-acquiring enzymes. In June, the investment in microbial C acquisition was greater in the epilimnia compared to the hypolimnia (mean vector length 8.9 vs. 4.7, $p = 0.02$). In July, however, this trend was no longer significant ($p = 0.54$), indicating distributed C acquisition of similar magnitude across lake strata in late summer.

3.3 Relationships between water chemistry and enzyme activities in lake epilimnia

Due to sampling constraints, DOC was measured in epilimnetic water only. There was a strong, linear increase in epilimnetic AP activity with increasing DOC concentration in June and July ($R^2 = 0.73$ and 0.84, respectively, p values < 0.01, Figure 6). BG activity was not related to DOC in June or July. Likewise, the activities of the N-acquiring enzymes, NAG+LAP, were unrelated to DOC in both seasons. None of the absolute EEAs were related to epilimnetic

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DIN:TP. When considered as enzyme ratios, epilimnetic investment in C:P acquisition (BG:AP) decreased with DOC concentration in June ($R^2 = 0.24$, $p = 0.01$) and was unrelated to DOC in July. C:N acquisition (BG:NAG+LAP) was unrelated to DOC in both months.

The magnitude of epilimnetic microbial P limitation, described by vector angles, increased with rising DOC concentration in both months (June and July $R^2 = 0.67$ and 0.57, respectively, p values < 0.01, Fig. 6). There were no relationships between vector angles and DIN:TP (data not shown). Likewise, there were no statistically significant relationships between vector length (i.e. C-limitation) and water chemistry (data not shown).

4 Discussion

Our results reveal a pattern of microbial P limitation across these southwest Greenland arctic lakes. Vector analysis indicated more severe P limitation in June compared to July, despite DIN:TP increasing in July. The overall pattern of P limitation of bacterioplankton and phytoplankton is consistent with previous research of plankton alkaline phosphatase activity in two lakes in the same study region, which also suggested P limitation of plankton communities (Brutemark et al. 2006). P limitation can be a factor that controls lake algal and microbial productivity and trophic status. In the High Canadian Arctic, for instance, P subsidies to lake and pond water caused increased microbial growth, indicating P as the primary limiting nutrient (Graneli et al. 2004). This was supported by another study completed across 20 lakes in Quebec, Canada, which demonstrated that TP, and not DOC, controlled microbial growth rates, respiration rates, and growth efficiency (Smith and Prairie 2004). Further, P availability controlled the fate of DOC, such that in oligotrophic, low-P concentration environments, DOC was mostly used for respiration (converted to CO_2), rather than being incorporated into biomass.

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Various measures of enzyme activity indicated a positive relationship between P limitation and DOC, contrary to our expectation that DIN:TP might be a stronger predictor. Here, we quantitatively measured and reported it as DOC, but for discussion, DOM is a more appropriate term as it includes organic N and P as well as carbon. DOM is a broad group of organic compounds with varying lability depending on the source, chemical structure, and N and P content (Mineau et al. 2013, Parr et al. 2015). In these lakes, DOM positively co-varied with TN but had no relationship with TP. Collectively, these enzyme and water chemistry data suggest that the DOM in these lakes may provide a readily available source of N, while higher DOM concentrations are associated with enzyme-mediated microbial P acquisition.

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DOM can contain distinct nutrient pools available for microbial consumption when conditions become stoichiometrically favorable. For instance, in a study of N-limited humic lakes in Northern Sweden, DOM-associated P was used by bacterioplankton and phytoplankton when N was added into the experimental systems (Jansson et al. 2001). Furthermore, in the same study the authors showed that bacterioplankton production was strongly controlled by DOC, such that bacterioplanktonic production in water containing 15-20 mg L^{-1} of DOC could not stimulated by further nutrient addition. However, DOM is not consistently a source of P in all lakes. Phosphorus amendments, in addition to simulated sunlight, were important in stimulating microbial degradation of DOC in an experiment using water from a southern Sweden humic lake, suggesting P limitation (Kragh et al. 2008). Another study of humic lakes located in southern Sweden demonstrated that P alone was not sufficient for stimulating microbial respiration and production; a source of labile C (glucose) was also required (Vidal et al. 2011). Together, these studies demonstrate that the interactions between bacteria and DOM are

complex—DOM can either behave as a source of bioavailable material, providing bacteria with energy and certain nutrients, or remain a recalcitrant, unavailable pool of organic compounds.

DOM remained an important factor in determining microbial P demand and limitation from June to July, suggesting DOM is the dominant source of nutrients available for microbial degradation and use. Lakes with low DOM appeared to have more seasonality of nutrient limitation than those with higher concentrations—as can be inferred from vector angles in Figure 7A, only low DOM lakes switched from P to N limitation in July, while higher DOM lakes remained consistently P limited. If the DOM pool is representative of N availability, it follows that N limitation would be more likely in low than in high DOM lakes. A seasonal shift in the type of DOM pool in low DOM lakes could also be contributing to seasonal differences in DOM-related nutrient dynamics. Crump et al. (2003) investigated bacterioplankton community dynamics in relation to DOM in Toolik Lake, Alaska. In spring DOM was flushed from the landscape into an inlet stream, and was labile due to extended soil and plant leaching, freeze-thaw processing, and microbial cell lysis. Moreover, this DOC was transported across the surface of the frozen tundra rather than the subsurface soils. The quality of this DOC then decreased as leaching of organic material decreased and the active layer deepened, allowing microbial degradation of DOM during transport.

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In some areas of the Arctic, discharge-normalized DOC export to arctic surface waters has decreased in recent decades (Striegl et al. 2005), with permafrost thaw and soil active layer deepening contributing to this trend (Carey 2003; Kawahigashi et al. 2004; Striegl et al. 2005; Striegl et al. 2007). If lakewater DOM concentrations and quality are also declining, it is likely that nutrient subsidy and limitation patterns will also change. Our data suggest that lakes receiving less DOC may become less P-limited and move towards N limitation, since DOM is

being suggested as an N source. Control of microbial production of enzymes and nutrient limitation may shift from organic matter to inorganic nutrients that are primarily flushed into the lakes during snowmelt and ice-off. Conversely, if DOC input into lakes were to change due to increased terrestrial production within catchments, lakes would be predicted to become universally P-limited. Changes in nutrients sources and concentrations will affect microbial and autotrophic productivity (Smith and Prairie 2004; Elser et al. 2007). It is likely that as the amount of lake DOM declines, the types of DOM qualities will shift as well. Bacterial community structure has been shown to change in correspondence with DOM quality in arctic lakes, as some bacteria prefer more labile compounds while other species are adapted to utilizing recalcitrant forms (Crump et al. 2003). In this study, the seasonal source and quality of the DOM pool might have been inferred by the inclusion of oxidative enzymes, such as phenol oxidase or peroxidase, which are responsible for degrading terrestrially-derived compounds such as phenols and aromatics, respectively (Sinsabaugh et al. 2008). Though BG is assumed to broadly represent C acquisition activity, oxidative enzyme activity may be an important metric in future studies.

Bacteria are of primary importance to freshwater ecosystems, as they transfer energy and nutrients often contained in organic matter to higher trophic levels (Azam et al. 1983), yet relatively little research on microbial ecology has been conducted in arctic lakes. It is therefore important to consider microbial responses to factors that will be changing in the near future (such as active layer depth and DOM concentrations) in order to understand ecological effects and directions of future change. We found that microbes in southwest Greenland lakes are generally P-limited, and that the strength of microbial P limitation decreased by mid-summer. Further, DOM was very important in determining microbial nutrient demands and limitation due to its potential as an N source. Lakes within the permafrost landscape of this study region are likely to

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experience shifts in nutrient limitation patterns as aquatic-terrestrial linkages potentially weaken (due to active layer increase) and DOM inputs decline. This study establishes current microbial nutrient limitation patterns that will allow us to assess response to future changes in this region.

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Table 1. Summary of epilimnetic physical and chemical data across the 24 lakes in June and July 2013. Significant changes ($p \leq 0.05$) between June and July means indicated by asterisk (*). Depth was not measured in July. S.E. = standard error.

	June			July		
	Mean	**S.E.**	**Range**	**Mean**	**S.E.**	**Range**
Depth (m)	19.7	1.67	8-36	-	-	-
pH	6.6	0.08	5.8-7.2	7.0*	0.07	6.4-7.7
Temperature (°C)	7.8	0.40	5.3-11.6	13.5*	0.35	9.1-17.4
DIN (µg L^{-1})	6	1.2	2-25	12*	1.1	4-22
TN (µg L^{-1})	467	47	178-1042	554*	61	197-1132
TP (µg L^{-1})	4	0.7	<2-12	5	0.7	<2-11
DOC (mg L^{-1})	12	1.9	4-35	13*	2.2	4-40
DIN:TP	2.1	0.37	0.17-6.0	5.4*	1.4	0.55-22

Table 2. Summary of Nutrient Limitation and C acquisition as indicated by mean vector angle and length for both months. Significant changes ($p \leq 0.05$) between June and July means indicated by asterisk (*). S.E. = standard error.

		June			July		
		Mean	**S.E.**	**Range**	**Mean**	**S.E.**	**Range**
Epilimnia	**Vector Angle (°)**	16	2.82	-8 - 41	4*	5.52	-32 - 41
	Vector Length	8.9	1.75	1.6 - 40.2	9.9	2.98	0.2 - 58.4
Hypolimnia	**Vector Angle (°)**	20	3.08	-6 - 45	15*	4.93	-30 - 43
	Vector Length	4.7	1.10	0.2 - 24.8	7.3	2.69	0.2 - 62.8

Figure 1. Map of study site, with the 24 study lakes surrounding Kangerlussuaq, Greenland indicated by dots.

Figure 2. An example of vector plot analysis for a hypothetical lake sampled in June and July. The 1:1 line is drawn in dashes and separates zones of P imitation (above) from N limitation (below). Vectors for each data point are drawn in arrows. Their angles indicate microbial nutrient limitation, such that the positive angle value with respect to the 1:1 line in June indicates P limitation, while the negative one in July indicates a shift to N limitation. The lengths of the vectors are also indicative of microbial C acquisition efforts, which in this example is greater in July than in June.

Figure 3. Absolute enzyme activities for: A) BG, B) NAG+LAP, and C) AP across epilimnia and hypolimnia of study lakes, from June to July. Error bars are standard deviation. Units of activity are µmol mL^{-1} hr^{-1}.

Figure 4. EEA ratios of BG relative to those of A) NAG+LAP and B) AP across lake strata for June and July. Error bars are standard deviation.

Figure 5. Scatterplot of microbial enzyme ratios (BG : NAG + LAP vs. BG : AP) about the 1:1 line. Included is C:P and C:N acquisition data of lake epilimnia (circles) and hypolimnia (triangles) from June (gray) to July (black). Dotted line indicates 1:1 (45 °) line. Vector angles (indicative of nutrient limitation) are calculated from these plotted data points, as deviation from the 1:1 line.

Figure 6. Response of A) AP, B) BG, and C) BG:AP to DOC in lake epilimnia. Data from June are indicated in grey triangles, data from July are indicated in black circles. Only significant relationships are displayed.

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Moved down [1]: Absolute enzyme activities for: A) BG, B) NAG+LAP, and C) AP across epilimnia and hypolimnia of study lakes, from June to July. Error bars are standard deviation. Units of activity are µmol mL^{-1} hr^{-1}

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Figure 7. Vector angles in response to A) DOC, and B) epilimnetic DIN:TP. Data from June are indicated in grey triangles, data from July are indicated in black circles. Dotted line indicates the boundary between P limitation (positive values) and N limitation (negative values). Only significant relationships are displayed.

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