Microbial activity in deeper mineral soil important for nitrogen cycling across successional stand age in a northern hardwood forest

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ABSTRACT

Little is known about soil processes with depth, as the majority of studies measuring soil nitrogen (N) cycling and microbial activity only measure the top 10-15 cm, where activity is expected to be highest. Microbes play an important role in the extraction of N from the mineral soil through the mineralization of organic N and in the accumulation of N in the mineral soil through immobilization and turnover of N in microbial biomass or in extracellular enzymes. It is also uncertain how these processes vary with successional stand age. To understand how N cycling varies with depth and forest stand age, we measured gross and net N mineralization and nitrification rates, as well as extracellular enzyme activity to 50 cm depth across three sites varying in stand age (45 and 100 years post-harvest and old growth) at and near Hubbard Brook Experimental Forest in central New Hampshire.

N- and C- degrading enzyme activity correlated with gross N mineralization rates, but not net N mineralization rate, directly linking microbial activity to the release of N from SOM. Enzyme activity and N cycling rates were expected to decrease with depth in response to decreasing soil C and N concentrations and increasing organic matter age. We found that N cycling rates per g dry soil and g N did decrease with between the forest floor and mineral soil, but decomposition enzyme activity and remained constant relative to decreasing organic matter concentrations with depth. On an areal basis, N cycling in the mineral soil was much larger, flux than in the organic horizon due to the large mass of the mineral soil. We found few differences in activity rates across sites, though net nitrification was significantly higher at the old growth site and enzyme activities were often lower at the 100 year old stand as compared the other two sites. The results from our study demonstrate the underappreciated importance of microbial activity with depth. Although N cycling rates decreased with depth, N turnover remained relatively high throughout the soil profile, highlighting the availability of N in old SOM and the likely importance of deep soil in supplying N to plants and microbes.

BIOGRAPHICAL SKETCH

Bridget Darby was introduced to biogeochemistry during her first year as an undergraduate at Boston University by her academic advisor Dr. Adrien Finzi. During her time as an undergraduate, Bridget was involved in biogeochemistry research both at Boston University with Dr. Finzi and during the summers at other institutions. In 2011, she participated in the Harvard Forest NSF REU summer program where she worked for Dr. Andrew Richardson of Harvard University, whose lab examines the spatial and temporal variation of vegetation phenology by using a network of digital cameras. In the summer of 2012, she participated in the Duke University Civil & Environmental Engineering REU, where she received training in molecular techniques used in microbiology while assisting a PhD student on his dissertation project in Dr. Claudia Gunsch's molecular biotechnology lab on the effects of antibiotics on denitrifiers in agricultural soil. Bridget completed her undergraduate honors thesis with Dr. Finzi entitled *'The coupled effects of carbon and nitrogen on soil decomposition: A theoretical model'*.

As an undergraduate, she was named a Goldwater Scholar and was awarded the Dean Elsbeth Melville Scholarship by Boston University for her involvement in research and high level of academic achievement. Bridget graduated from Boston University in 2013 summa cum laude with a B.A. in biology and minor in mathematics and received the Senior Book Award in Biology.

After receiving her B.A., Bridget began graduate school at Cornell University in the field of Ecology and Evolutionary Biology, studying terrestrial biogeochemistry with Dr. Christine Goodale. She was awarded a National Science Foundation Graduate Research Fellowship and a

Cross-Scale Biogeochemistry and Climate IGERT traineeship to fund her graduate studies. While at Cornell, she participated in the Biogeochemistry, Environmental Science and Sustainability (BESS) graduate student association and the Cross-Scale Biogeochemistry and Climate IGERT. Through these programs, she was able to form collaborations with scientists at the National Center of Atmospheric Research, Cary Institute for Ecosystem Research, and with a microbiology lab at Cornell, all of which contributed to her thesis research.

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Introduction

Studies measuring soil microbial N cycling and enzyme activity often focus exclusively on the carbon-rich forest floor and surface mineral horizons, typically to 10-15 cm depth, where microbial activity is expected to be highest. Much less is known about microbial activity in the deeper mineral soil, where cycling rates are expected to be lower due steep declines in C and N content and assumed decreases in substrate quality (Booth et al. 2005;). Nonetheless, a global data set indicates that over 60% of soil organic N and NO_3^- in the top meter of soil is located below 20 cm (Jobbagy and Jackson 2001). The few studies of microbial processes in deep soil have found that they are still detectable at depths > 1 m, but that microbial biomass and extracellular enzyme activity per unit soil mass decline with depth (Taylor et al. 2002; Fierer et al. 2003; Morse et al. 2014; Stone et al. 2014). However, the great mass of the mineral soil pool means that microbial activity at depth could contribute meaningfully to ecosystem-scale processes despite these lower rates.

Microbial processes can both remove and add N to soil organic N pools. First, some microbes drive the process of N mineralization, or the release of N from soil organic matter (SOM), by their production of a range of extracellular enzymes (e.g., Schimel & Bennett 2004). These enzymes degrade various SOM constituents such as cellulose (e.g., β-glucosidase, cellobiohydrolase), lignin and related compounds (phenol oxidase and peroxidase), chitin (N-acetyl glucosaminidase), and proteins (leucine aminopeptidase). Microbes can remove N from the soil by immobilizing available organic N and incorporating this N into their biomass.

The potential activity of N-degrading enzymes may serve as a metric for N mineralization, but directly measuring gross N transformation rates is the only way to quantify the overall microbial production and consumption of inorganic N (Davidson et al. 1991, Hart et

al. 1994). Information on gross N cycling can provide a mechanistic understanding of soil N dynamics, but the ^{15}N pool dilution technique used to measure these processes is complicated and relatively expensive, and so many studies use various simpler assays of net N mineralization and nitrification (Bohlen et al. 2001; Schimel and Bennett 2004; Robertson et al. 1999). Net rates represent the balance between the production and consumption of inorganic N by microbes. The direct measurement of gross rates of mineralization and nitrification can provide additional insight into N cycling beyond what net rates have shown. For example, Davidson et al. (1992) found large gross nitrification and NO_3^- consumption rates in a mature coniferous forest despite low net nitrification rates, showing that NO_3^- plays a more important role in that forest's N cycling than the net rates would suggest.

The Hubbard Brook Experimental Forest (HBEF), a Long-Term Ecological Research site in central New Hampshire with a long history of biogeochemical monitoring, serves as a unique location to study microbial N cycling at depth and over succession. Long-term biogeochemical monitoring at Hubbard Brook has revealed unexpected changes in N balance (Yanai et al. 2013). Ecological theory suggests that ecosystem nitrogen retention should be positively correlated with biomass accumulation of the ecosystem, meaning young forests with high rates of biomass accumulation should have high net N retention rates, whereas mature forests should retain little N (Vitousek and Reiners 1975). Yet, stream export at HBEF has decreased with time despite steady N inputs from atmospheric deposition, reduced N accumulation in live biomass, and unchanging N accumulation in coarse woody debris and forest floor pools (Yanai et al. 2013). The fate of this missing N must be either N accumulation in the mineral soil or N gas loss (Yanai et al. 2013; Dittman et al. 2007).

Plants can mine N from the mineral soil during early phases of regrowth (Johnson 1992), through stimulating N mineralization through allocation of photosynthate to mycorrhizal symbionts (Hobbie and Hobbie 2006) and by priming of bacterial decomposition activity with exuded labile C compounds (Kuzyakov et al. 2000). As biomass accumulation slows, soils of old-growth stands may then accumulate N that is no longer accumulating in plant biomass (Kaye et al. 2003). Therefore, we would expect changes in enzyme activity and N cycling to reflect these changes.

This study addressed how microbial N cycling and extracellular enzyme activity varied with depth and forest stand age at and near the HBEF. Understanding how N cycling and mining differ with stand age throughout the mineral soil can help to identify the microbial mechanisms controlling N extraction and accumulation in mineral soil at HBEF. We measured gross and short-term net N transformation rates and potential extracellular enzyme activity to 50 cm soil depth at three sites: two sites at the HBEF that varied in stand age (45 and 100 years post-harvest) and at an old-growth site at nearby Mt. Moosilauke. We hypothesized that 1) gross N mineralization rates would correspond with the activity of associated decomposition enzymes; 2) enzyme activity and N transformation rates would both decrease with depth in response to decreasing soil C and N concentrations; 3) enzyme activity and gross N mineralization rates would be highest in the youngest site as a result of priming driven by plant demand for N, while the old growth site would have highest net nitrification rates due to lower competition for N by plants.

Methods

Site Description:

Soils were collected from three sites with stand age ranging from 40 to >200 years at the Hubbard Brook Experimental Forest (HBEF) and nearby Mt. Moosilauke (Table 1). HBEF is a temperate mixed hardwood forest in central New Hampshire with a 50-year record of N precipitation input and stream output measurements (Likens and Bormann 1995). The soils are largely sandy loam Spodosols derived from glacial till and are categorized as well-drained Haplorthods. The forest has a mean temperature of 18°C in July and -9°C in January, and receives about 1,400 mm of precipitation, approximately one third of which is snow. Soil sampling at Hubbard Brook occurred within Watershed 4 (W4), which was strip cut in three phases between 1970 and 1974, and at a site west of Watershed 6 (WW6), the biogeochemical reference watershed at HBEF, which was heavily logged between 1906 and 1920 (Peart et al. 1992). The WW6 site corresponds to the mid-elevation site as described in other studies at the HBEF (Bohlen et al. 2001; Dittman et al. 2007). The Mt. Moosilauke old-growth site (MMOG) is located ~20 km away from HBEF and had a similar species composition and soil type to those at HBEF. Tree ring data confirm that some trees are >200 years (Keurajian 2015). Streamwater and some soils from this site were sampled by Vitousek (1977) with streams resampled by Goodale et al. (2003). The forest at both WW6 and MMOG is dominated by sugar maple (*Acer saccharum*) whereas the forest in W4 is dominated by yellow birch (*Betula alleghaniensis*) (Table 1).

Field and Laboratory Methods:

Eight deep soil cores (0-50 cm) were collected at each site during the first two weeks of July, 2014, of which half were randomly selected for the microbial analyses described here. The forest floor (Oe/Oa) was collected by placing a 15 x 15cm wooden frame on the top of the Oe horizon and then using a knife to cut out a block of the Oe and Oa horizons. A diamond bit

rotary corer (Rau et al. 2011) with a 9.5 cm internal diameter was then used to quantitatively collect the mineral soil in 10 or 20 cm increments to 50 cm depth (0-10, 10-20, 20-30, and 30-50 cm). Bulk density, and soil C and N stocks were determined using the quantitative samples (data not shown) and these values were used to calculate rates per g N or C. Soils were stored on freezer packs during transport and at 4 °C in the laboratory until processing. Soils were sieved to 4 mm within 24 hours of collection. A coarse sieve was chosen in order to minimally disturb soil aggregates, while still homogenizing the sample and removing larger roots and rocks. Sub-samples of the sieved soil were stored at -20 °C for later use in enzyme activity assays. The remaining soil was stored at 4 °C for up to 2 more days until used for gross N cycling assays.

Separate 10 g soil subsamples were taken for moisture determination after drying for 1 day at 110 ^{o}C and for C and N concentration and isotopic composition after grinding to a fine powder using a ball mill (Retsch mixer mill MM200; Verder Scientific, Newtown, Pennsylvania, USA). Soil C and N concentration and isotopic composition were measured at the Cornell Stable Isotope Lab, as described above.

Gross rates of N mineralization and nitrification rates were assessed using the isotope dilution method (Davidson et al. 1991; Hart et al. 1994). In this method, a known amount of $^{15}NH_4^+$ or $^{15}NO_3^-$ is added to soil samples, and the rate of dilution of this ^{15}N by mineralization or nitrification of unlabeled N over 24 hours is measured and used to calculate gross rates of these processes. This method assumes no internal recycling of N during the assay. To estimate rates of gross N mineralization and $^{15}NH_4^+$ consumption, 7.53 µg N of 98 atom% $(^{15}NH_4)_2SO_4$ was added to each of two 15 g field moist sub-samples from each soil depth at each site in 125 mL HDPE bottles. The label was distributed within the soil sample in 1 mL of deionized water using a 250 µg Hamilton syringe.

One of the sub-samples was extracted with 50 mL of 2M KCl within 15 minutes of labeling. The second sub-sample was incubated in the dark for 24 hours at room temperature before being extracted with KCl. The KCl extracts were stored in 60 mL HDPE bottles and frozen at -20°C until chemical analysis. The same procedure was used to estimate rates of gross nitrification and $^{15}NO_3^-$ consumption, using 1.63 μg N of 98% $K^{15}NO_3^-$ per sample (instead of $(^{15}NH_4)_2SO_4$). These ^{15}N additions were based on previous measurements of gross N cycling rates at other northern hardwood forests with similar soil inorganic N concentrations (Christenson et al. 2009; Fisk et al. 2002). The label increased the extractable inorganic N pool size by ~2-35%, depending on the sample.

After extraction, samples were analyzed for NH_4^+ and NO_3^- concentrations colorimetrically using a Quikchem 8100 flow injection analyzer (Lachat Instruments, Milwaukee, WI) at the Cary Institute for Ecosystem Studies. The N diffusion method (Brooks et al. 1989) was then used to determine ^{15}N in the NH_4^+ or NO_3^-. A sealed Teflon tape packet containing two small glass fiber filters that were acidified with 20 uL $KHSO_4$ was floated on the KCl extract in a 125 ml HDPE bottle. MgO was added to both $^{15}NH_4^+$ and $^{15}NO_3^-$labeled samples to increase pH and convert NH_4^+ to NH_3 which volatilizes and is trapped on the acidified filters. Immediately after adding MgO, Devarda's alloy was added to the $^{15}NO_3^-$ labeled samples to convert NO_3^- to NH_4^+, such that both NH_4^+ and NO_3^- were collected on the acidified discs in these samples. The ^{15}N contents of the acidified discs were analyzed at the Cornell Stable Isotope Laboratory in Ithaca, NY using a Finnigan MAT Delta Plus mass spectrometer following combustion with an elemental analyzer (Carlo Erba NC2500; Thermo Finnigan, San Jose, CA, USA).

Gross N cycling rates were calculated using the difference in atom percent ^{15}N above background (APE) and the difference in inorganic N concentrations between the pre- and post-incubated samples using the equation in Hart et al. (1994), originally developed by Kirkham and Bartholomew (1954):

$$m = \frac{[NH_4^+]_0 - [NH_4^+]_t}{t} * \frac{\log(APE_0/APE_t)}{\log([NH_4^+]_0/[NH_4^+]_t)}$$

$$c = m - \frac{[NH_4^+]_t - [NH_4^+]_0}{t}$$

Where m = the gross mineralization rate, APE = the ^{15}N atom percent excess in the NH_4^+ pool, c = the gross NH_4^+ consumption rate, t = incubation time, and $[NH_4^+]$= the total extractable NH_4^+-N concentration at time 0 or t. Gross rates of nitrification and NO_3^- consumption are calculated using the same equation, but replacing $[NH_4^+]$ with the total inorganic N concentration (NO_3^--N + NH_4^+-N) and using the ^{15}N atom percent excess in the total inorganic N pool. The total inorganic N pool is used instead of NO_3^- to calculate gross nitrification and consumption because both NH_4^+ and NO_3^- are collected on the acidified discs during the diffusion process for the $^{15}NO_3^-$ samples, as described above.

One-day net N mineralization and nitrification rates were calculated using the pre- and post- incubation extractable inorganic N measurements collected during the gross N mineralization assay. Net N mineralization was calculated as $([NH_4^+\text{-N}]+[NO_3^-\text{-N}])_{incubation}$ - $([NH_4^+\text{-N}]+[NO_3^-\text{-N}])_{initial}$ and net nitrification was calculated as $[NO_3^-\text{-N}]_{incubation}$- $[NO_3^-\text{-N}]_{initial}$. Rates reported here include values computed per g dry soil, per g soil N, and on an areal basis, which was calculated by multiplying values per g dry soil by the mass of dry soil measured in the quantitative soil core samples (cf. Rau et al. 2011).

Extracellular Enzyme Activity

Fluorometric assays were used to quantify the activity of different enzymes used for microbial acquisition of C-, N- and P from SOM at each of the sites by soil depth. The following hydrolytic enzymes activities were measured using the method outlined in German et al. (2011): N-acetyl glucoaminidase (NAG), leucine aminopeptidase (LAP), β -glucosidase (BG), cellobiohydrolase (CB), β -xylosidase (BX), and acid phosphatase (AP) (Table 2). This method can only measure potential enzyme activity and therefore cannot provide information on *in situ* rates or enzyme production or turnover (Wallenstein and Weintraub 2008). However, this assay is useful in comparing relative rates across samples. Briefly, frozen soil sub-samples stored at -20°C were thawed and 2-3 g were added to 150 mL of acetate buffer (pH=5). The solution was homogenized using a blender to make a soil slurry; 50 uL was added to 8 replicate wells in a column of a 96-well plate. The slurry was then incubated in the dark at room temperature for a fixed time after adding a fluorescent substrate specific to each enzyme function (Table 2). The fluorescence is measured on a microplate reader set at 365 nm excitation and 450 nm emission. Assays were run alongside a standard curve containing soil homogenate with an increasing concentration of methylumbelliferone (MUB). Fluorescence is converted to units of potential enzyme activity (nmol h^{-1} g^{-1}) as in German et al. (2011).

Statistical analysis:

Data were analyzed using R statistical software. Data transformations using a log transformation were considered if data was not normally distributed; however, if statistical relationships remained the same regardless of transformation, the non-transformed data was used for final analysis. A two-way ANOVA was used to determine differences in N-cycling rates and enzyme activity with depth and across the three sites. Tukey's tests were used for post-hoc comparisons. Correlations were tested using Pearsons' correlation coefficient.

Results:

Soil properties:

Soil C and N concentrations decreased exponentially with depth (Figure 1 a-b). The forest floor had significantly higher C and N concentrations than the mineral soil ($p < 0.001$), and N concentrations in the surface mineral soil (0-10 cm) were significantly higher than the deeper mineral soil (10-50 cm) for all of the sites ($p < 0.001$). There were no significant differences in C and N concentrations across sites ($p=0.21$, $p=0.59$ respectively), though WW6 trended toward higher C concentrations in the deeper mineral soil (20-50 cm) relative to the other two sites. Soil C:N ratio declined from the organic horizon to 0-10 cm depth (Figure 1 c). At MMOG and WW6, soil C:N ratio increased again with 10-50 cm depths, while soil C:N ratio did not change with depth at W4. WW6 had the highest C:N ratio (≥ 20 at all depths) of the three sites for all depths and this difference was significant between 20-50 cm depth ($p < 0.05$). MMOG had the lowest C:N ratio for all depths (C:N < 18 for all mineral soil depths).

Gross N cycling rates

Gross N mineralization and NH_4^+ consumption rates per g dry soil declined significantly between the organic horizon and mineral soil for all three sites ($p < 0.001$) (Figure 2 a; NH_4^+ consumption not shown, as it can be calculated by the difference in gross and net N mineralization). Gross N cycling rates per g soil did not differ by depth within the mineral soil ($p > 0.05$ for all depths) and did not differ across the three sites within depths ($p > 0.05$ for all sites). When gross rates per g N were pooled across sites there was a significant difference ($p < 0.001$) with depth between the organic horizon and mineral soil for both gross N mineralization and NH_4^+ consumption. There was no significant site × depth interaction for gross N mineralization ($p=0.45$) or NH_4^+ consumption ($p=0.69$) per g N, but post-hoc comparisons

revealed some differences with depth for individual sites (Figure 2b; NH_4^+ consumption not shown). Gross N cycling rates per g N were significantly higher at MMOG than WW6 ($p=0.002$).

Unfortunately, gross nitrification and NO_3^- consumption rates could not be reported because post- incubation samples labeled with NO_3^- were more enriched in ^{15}N than the corresponding pre-incubation samples. As the name of the method suggests, the ^{15}N label in post-incubation samples should be diluted relative to pre-incubation samples when using the ^{15}N dilution method. Not only does our result suggest an issue with the method, but in many cases the increase in enrichment also yielded rates to be very low to negative. The cause of this methodological issue is unknown and has not been explored in the literature. However, studies in similar ecosystems have reported low, and sometimes negative values for gross nitrification and NO_3^- consumption (Ross et al. 2004; Christenson et al. 2009; Fisk et al. 2002).

Net cycling rates

Net N mineralization rates were ~40-90% lower than the gross N mineralization for each site and depth, with the exception of WW6 30-50 cm depth in which net N mineralization was twice as high as the gross rate (Fig. 3). The net N mineralization rate at 30–50 cm depth at WW6 may be higher than the gross rate because net N mineralization calculations include nitrification and ammonification and nitrification dominates net N mineralization for 30-50 cm at WW6. Net N mineralization and nitrification rates per g dry soil significantly declined between the organic and mineral soil just as with the gross cycling rates ($p < 0.0001$) (Figure 3 a-b). As with the gross N cycling rates, net cycling rates per g N decreased significantly with depth when pooled across sites ($p \leq 0.0002$) (Figure 3 c-d). Net N mineralization rate per g N was significantly higher in the organic horizon than the 10-50 cm depths and net nitrification rate was significantly higher in

the organic horizon than the 10-50 cm depth and higher in 0-10 cm than the 20-50 cm depths. There were no significant differences by site in net N mineralization rates (per g dry soil and g soil N) ($p = 0.27$, $p = 0.2$ respectively), and although there was a significant site effect for net nitrification (for per g dry soil and g soil N) ($p < 0.001$ for both units), the only difference driving this effect was in the organic horizon, where MMOG had more rapid nitrification than the other two sites ($p < 0.001$). There was a significant site x depth interaction for both net N mineralization ($p < 0.001$) and net nitrification ($p < 0.001$) per g N, but the interaction was only net nitrification per g dry soil ($p=0.002$) (Fig 3).

Across all samples, neither gross nor net N cycling rates correlated significantly with soil C:N ratio ($p>0.05$ for all rates). When separated by site, MMOG gross and net N cycling rates were positively correlated with soil C:N (gross N mineralization: $r= 0.68$, $p<0.01$; NH_4^+ consumption: $r=0.597$, $p<0.01$; net N min: $r= 0.69$, $p<0.001$; net nitrification: $r=0.56$, $p<0.001$), but rates were not correlated with soil C:N at the other sites.

Ecosystem scale

On an areal basis, N cycling in the mineral soil becomes an equivalent, if not larger, flux than in the organic horizon due to the large mass of the mineral soil (Figures 4 a-d). In general, the 0-10 cm depth had the highest flux of both net and gross N cycling, although this difference was only significant for gross N mineralization, NH_4^+ consumption at MMOG and net nitrification at MMOG and W4. There were no differences across sites within each depth for all of the area based N transformations. MMOG had higher gross N mineralization and NH_4^+ consumption rates than WW6 and this difference was the only significant site difference between total profile cycling rates.

Enzyme activity

All potential extracellular enzyme activity declined with depth when normalized per g dry soil (figure not shown), as with the N cycling rates. However, when considered per g soil C, for AP, BG, and LAP activity tended to remain relatively constant with increasing depth (Figure 5 a-f). BX activity per g C was significantly higher between 0-20cm than the organic horizon ($p = 0.003$), and CB and NAG activity per g C declined significantly between the organic horizon and mineral depths ($p < 0.0001$ for both enzymes). WW6 usually had significantly lower activity per g C at each depth than MMOG or W4 ($p > 0.05$). For individual extracellular enzymes at each site, all considered per g soil C, AP did not vary significantly with depth at any site (Figure 5a). BG activity was significantly higher in MMOG than WW6 at every depth, but few differences existed between W4 and these two sites. In general, BG activity did not vary significantly with depth by site ($p = 0.1$). BX activity at WW6 was significantly lower than W4 or MMOG for all depths ($p < 0.0001$) (Figure 5c), but BX activity did not have a significant depth x site interaction ($p = 0.08$). CB activity was significantly higher at MMOG in the organic horizon than the mineral soil ($p < 0.001$), and the organic horizon at MMOG was also significantly higher than the organic horizons at W4 and WW6 ($p < 0.0001$) (Figure 5d), but there were no other significant differences in CB activity across sites or with depth for each site. There were significant differences in NAG activity with depth for MMOG and WW6 ($p < 0.05$ for depths at each site) (Figure 5e). WW6 had significantly lower LAP activity than MMOG for all depths ($p < 0.0001$), but there were no significant differences in LAP activity with depth ($p=0.22$) (Figure 5f).

There was a significant positive correlation between NAG activity and gross N mineralization ($r = 0.66$, $p < 0.001$) (Figure 6a) and NAG activity and total C-degrading enzyme activity, the sum of BG+CB+BX ($r = 0.56$, $p < 0.001$) (Figure 6b). There was no correlation

between net N mineralization and NAG activity (r =-0.05, p = 0.69) (Figure 6c) and a non-significant negative correlation between total C-degrading enzyme activity and net N mineralization (r = -0.18, p=0.17) (Figure 6d).

Discussion:

Gross N mineralization and enzyme activity

The strong correlation between NAG enzyme activity and gross N mineralization directly links the release of N from SOM to the conversion of organic N forms to inorganic forms. This result agrees with our hypothesis that enzyme activity and gross N mineralization should be functionally related (e.g., Schimel & Bennett 2004), connecting the overall process of N acquisition. As such, we would not expect a relationship between enzyme activity and net mineralization given that the net value represents the difference between inorganic N production and consumption. Chitin is considered a major source of organic N in soils and the hydrolysis of chitin to amino acids by NAG acts as a major source of mineralizable N in soils (Stevenson 1994). Ekenler and Tabatabai (2002) found that NAG activity positively correlated with net N mineralization at two agricultural sites and concluded that NAG activity could be a rate-limiting step in N mineralization. To our knowledge, only one other study has related enzyme activity with N cycling and reported a positive relationship between gross N mineralization and enzyme activity, but this study measured a different suite of enzymes as we have, instead measuring deaminase, protease, and urease (Zaman et al. 1999). Similar to NAG, the extracellular enzymes measured in Zaman et al. (1999) target organic N compounds.

Depth

We found that organic horizon and surface mineral soil net N cycling rates were broadly similar to those previously measured at HBEF (Bohlen et al. 2001; Groffman et al. 2009) and

gross N mineralization rates resembled those measured previously across similar hardwood sites in the Northeast U.S. (Christenson et al. 2009; Fisk et al. 2002; Ross et al. 2004; Booth et al. 2005). This study adds to these existing measurements by characterizing gross N cycling patterns with depth, showing that deep soils cycle as much, and sometimes more N as surface soils.

We originally hypothesized that N cycling and enzyme activity rates would decline with depth as a function of decreasing C and N concentration. We found that these patterns occurred as expected for both N cycling rates and enzyme activities when examined per g dry soil, with decreasing rates or activities with increasing depth. We also found that there was a decrease in N cycling rates per g N between the organic and mineral soil when sites were pooled. Unfortunately, we did not have the statistical power to detect differences in N cycling per g N with depth within a site, because of a limited to sample size and high variation across replicate cores. Unexpectedly, we found that enzyme activity per g C did not decrease with depth with the exception of NAG and CB.

In a synthesis of gross and net N cycling measurements across forest, shrubland, grassland and agricultural ecosystems, Booth et al. (2005) found mineralization rate to correlate with soil %C and %N, properties that decline sharply with depth (Fig. 1). That synthesis was limited to studies examining the top 15 cm of soil, but in a study of net N and C cycling with depth at HBEF across a range of hydropedologic settings, Morse et al. (2014) found that correspondence between potential net N mineralization per g soil and soil C concentration also applied to deeper soils.

SOM is expected to be older, more processed and more stable with increasing depth. For example, a ^{14}C study at Bartlett Forest, a hardwood forest in New Hampshire within 25 km of

HBEF, revealed that mean bulk turnover time increased with depth between the forest floor and mineral soil and within the mineral soil itself, increasing from ~90 years in the Oe/Oa to ~400 years at 5-15 cm depth to ~900 years at 30-45cm depth and that turnover time was longest for the heavy fraction (McFarlane et al. 2012). Another ^{14}C study at Harvard Forest, Massachusetts reported similar turnover times for C with depth as observed in New Hampshire, and a found that SOM age and turnover time increased in all soil fractions with depth (Gadunski et al. 2000). The few studies of net N cycling with depth in forest soils have found net N cycling rates to decline by at least an order of magnitude between the forest floor and B horizon (15 – 50 cm) on both a per g dry soil and per g OM basis, but that rates did not change with depth within the mineral, consistent with our result (Federer 1983; Persson et al. 1995). ^{14}C studies can only capture bulk age, therefore it is possible that amount or proportion of young, reactive SOM remains relatively constant with depth, which could partially explain the constant rates of cycling we observed in the mineral soil.

In addition to the above changes in organic matter quantity and age with depth, plant-derived, cellulolytic compounds have been found to decrease with depth in rye and corn plots in a long-term agricultural field experiment in Germany (Kramer and Gleixner 2008). Activity of enzymes that target these compounds, such as β-glucosidase (BG) or cellobiohydrolase (CB) likely follow a similar pattern. Of the few studies of decomposition enzyme activity with depth, most report rates on a per g dry soil basis and report that rates decline with depth, most dramatically between the forest floor and mineral soil (Taylor et al. 2002; Herold et al. 2014). It is less common to report rates per g C or g OM. In a study of enzyme activity at a tropical forest site, Stone et al. (2014) also found that C and N-degrading enzyme activity per g C did not vary with depth (to 140 cm) and hypothesized the high rates of enzyme activity throughout the soil

profile could be due in part to microbes producing enzymes continuously at low levels regardless of conditions (i.e. constitutive enzyme activity). It has been shown that microbes produce many enzymes at low constitutive levels, unless stimulated by an environmental signal (Allison et al. 2011). Enzymes may also persist at depth due to increased mineral stabilization. Soil enzymes can be stabilized on soil minerals by covalent bonding or adsorption (Burns et al 1982; Taylor et al. 2002) and this stabilization decreases degradation as compared to free enzymes (Ladd 1978; Allison et al. 2006). In addition to total mineral content increasing between the forest floor and mineral soil, Al- and Fe- containing minerals accumulate in the spodic horizon (in our study the spodic horizon location was between 5-50 cm depending on the core). Allison et al. (2006) showed that the addition Al- and Fe- containing minerals to soils increased enzyme stability and decreased enzyme degradation.

Our results of high rates of N cycling per area with depth demonstrate the important role of the deeper mineral soil when considering soil N cycling. Had we only measured the forest floor and top 10 cm of mineral soil, we would have missed ~40-50 % of the whole-profile N mineralization and nitrification. The few studies of gross or net N cycling with depth confirm our results, reporting about 50% of total gross or net N mineralization occurring below the top 15 cm on a per area basis (Iverson et al. 2011; Morse et al. 2014). As at our sites, the top 10 cm of mineral soil was found to have the largest flux of gross and net N mineralization (Iverson et al. 2011; Morse et al. 2014). The top 10 to 15 cm of mineral soil is likely to dominate total ecosystem flux because of the combination of %OM, higher mineral content and higher root biomass (Park et al. 2008), which contributes to SOM formation.

Site differences

We found few differences in gross or net N mineralization differed with stand age. Our hypothesis that net nitrification would be highest at the old growth site (MMOG) was partially supported, as MMOG had higher net nitrification rates in the organic horizon, but not the mineral horizons. Net nitrification rates in sugar maple-dominated old-growth forests elsewhere in the White Mountain National Forest, NH, were found to be nearly double those in sites that had been logged or burned within the last 100 years, but net mineralization was found to be the same regardless of land-use history (Goodale et al. 2001). This difference in net nitrification was attributed in part to a lower C:N ratio in the old growth forest floor and mineral soil. Soil C:N has been determined to be a predictor of net nitrification in northeastern forests, with a critical C:N threshold for N mineralization and nitrification of 20-24 (Aber et al. 2003; Ollinger et al. 2002; Lovett et al. 1999; Mooshammer et al. 2014). In our study, MMOG had much lower soil C:N ratio for all mineral soil depths and was the only site where N cycling rates were correlated with soil C:N, which could be leading to higher nitrification rates at this site. In contrast, net nitrification did not vary with stand age in a sugar maple dominated old-growth stands in Michigan and old-growth conifer stands in California (Davidson et al. 1992; Fisk et al. 2002). Both of these old-growth stands had higher gross N immobilization than secondary growth stands, and found that this difference corresponded to lower gross nitrification (Davidson et al. 1992; Fisk et al. 2002). Neither study reports soil C:N ratio so it is difficult to know if it influenced nitrification rates at these sites, but likely species differences in litter composition could perhaps partially explain differences between these studies and our results. Although the Michigan sites were dominated by sugar maple, hemlock was a secondary species at the site and Douglas fir dominated the sites in California. Sugar maple stands often have high rates of gross

and net nitrification, whereas nitrification rates under hemlock are often very low (Lovett et al. 2004; Finzi et al. 1998; Christenson et al. 2009).

Our hypothesis that extracellular enzyme activity would be highest at the youngest site (W4) as a result of priming driven by plant demand for N was not supported, as hydrolytic enzyme activity at W4 was usually not significantly different from either of the other sites (Fig. 5) ($p > 0.05$), but fell intermediate between the two older sites, WW6 and MMOG. We also did not expect the lowest enzyme activity rates (per g C) to occur at the mid-aged site, WW6. Hydrolytic enzyme activity corresponded well with gross N mineralization rates (Fig. 6), but priming activity may be better reflected though measurements of the activity of oxidative enzymes, such as phenol oxidase and peroxidase, which facilitate the breakdown recalcitrant SOM. Because decomposing recalcitrant SOM to gain access to N has a high C cost (Sinsabaugh 2010; Moorhead and Sinsabaugh 2006), we would expect the highest oxidative activity when plant N demand is highest.

It is likely that differences other than age between sites obscured possible age effects. For example, W4 is birch dominated, whereas MMOG and WW6 are dominated by sugar maple. Tree species differences can affect soil N cycling through differences such as litter quality, mycorrhizal association (Finzi et al. 1998; Lovett et al. 2004). WW6 also had notably different soils than the other two sites, with more C at depth. Although it can be difficult to control for site differences in species composition, parent material, and C and N stock, future work to expand the chronosequence to include more stand age replication could improve statistical power so that an age effect could be detected.

This study demonstrates the often underappreciated importance of microbial activity in deep soils. Gross N cycling rates corresponded with potential rates of hydrolytic extracellular

enzyme activity, demonstrating a tight link between microbial decomposition processes and the release of N from SOM, across sites and soil depths. We found large decreases in soil N cycling rates per g soil with increasing depth, but these differences were more than compensated by the large size of the mineral soil pool. Nonetheless, N turnover remained relatively high throughout the soil profile, highlighting the availability of N in old SOM and the likely importance of deep soil in supplying N to plants and microbes.

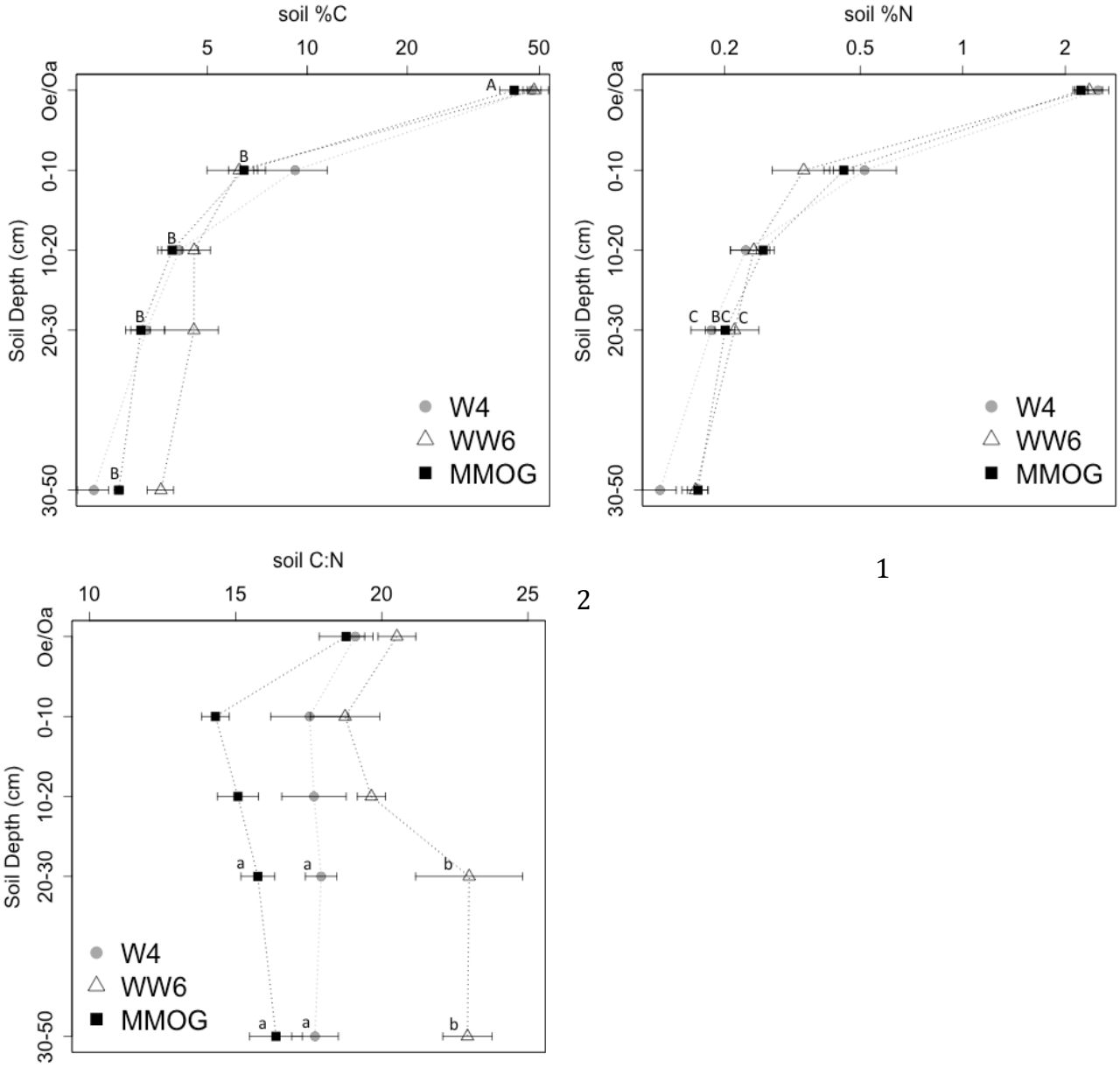

Figure 1. Soil properties with depth for the three site: %C, %N and soil C:N ratio. Different letters denote significant differences. Upper-case letters denote significant differences between depths within a site. Lower-case letters denote differences across sites within a depth. The absence of letters indicates non-significant relationships. Error bars represent standard errors of the mean (n=4 at each site).

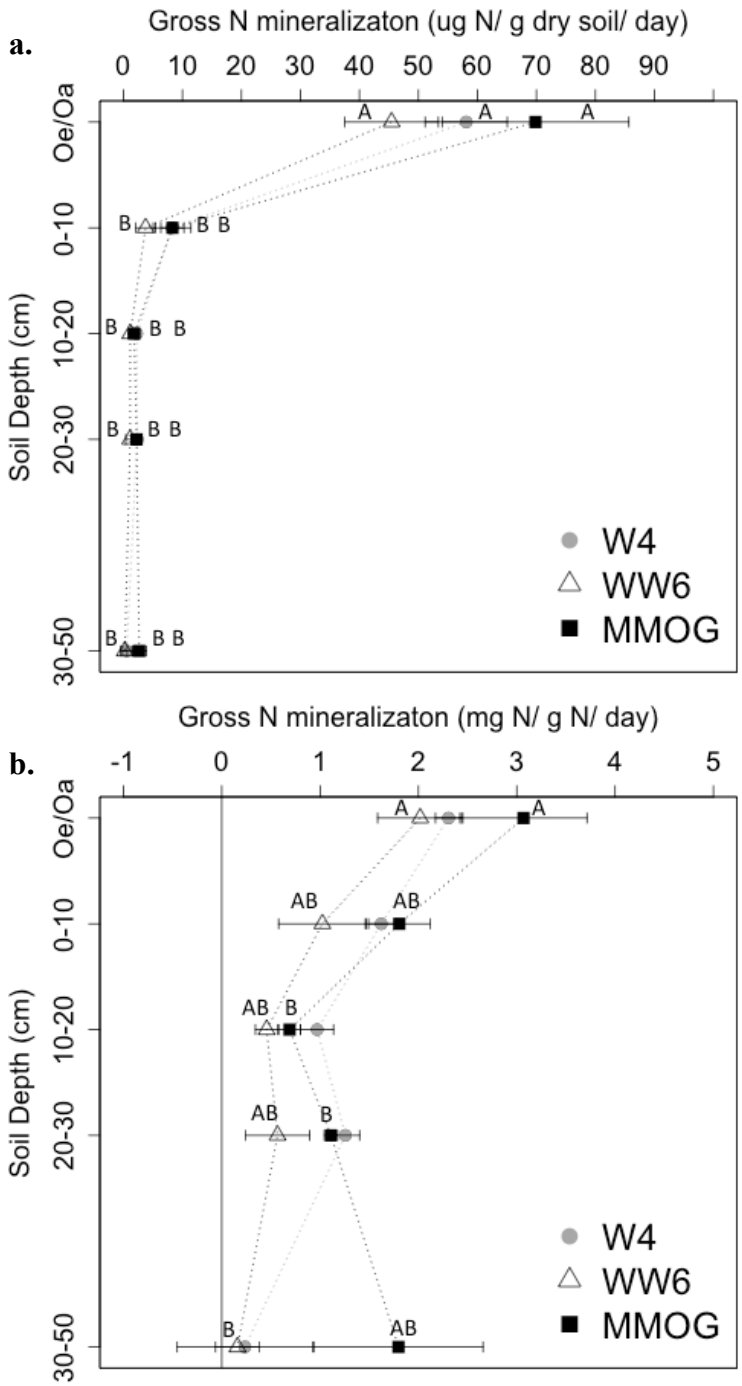

Figure 2. Gross N mineralization rates **a.** per g dry soil and **b.** g soil N with depth for the three sites. Different letters denote significant differences. Upper-case letters denote significant differences between depths within a site. Lower-case letters denote differences across sites within a depth. The absence of letters or symbols indicates non-significant relationships. Error bars represent standard errors of the mean.

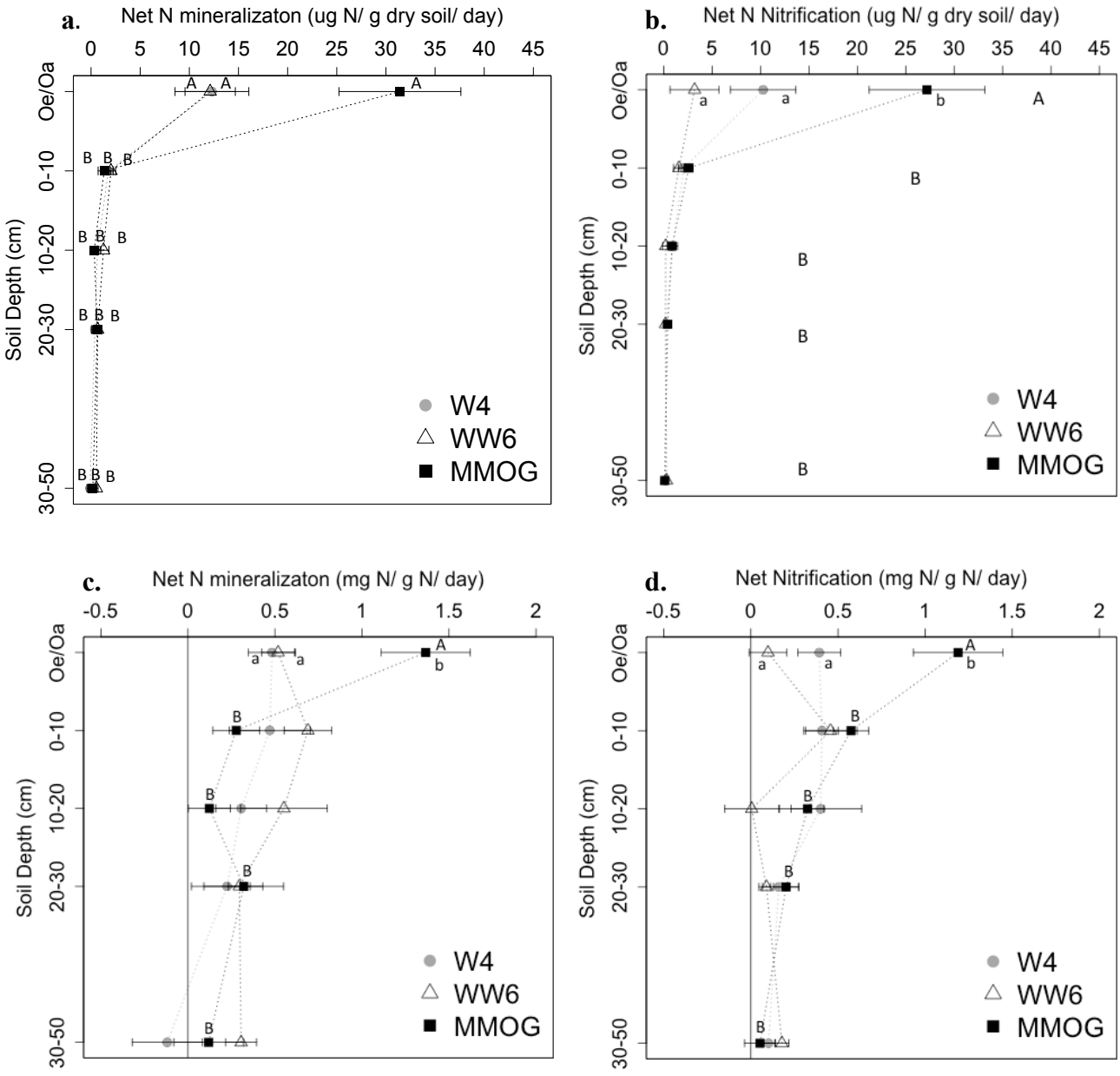

Figure 3. Net N mineralization and nitrification rates per g dry soil (a-b) and g soil N (c-d) with depth for the three sites. Different letters denote significant differences. Upper-case letters denote significant differences between depths within a site. Lower-case letters denote differences across sites within a depth. The absence of letters indicates non-significant relationships. Error bars represent standard errors of the mean.

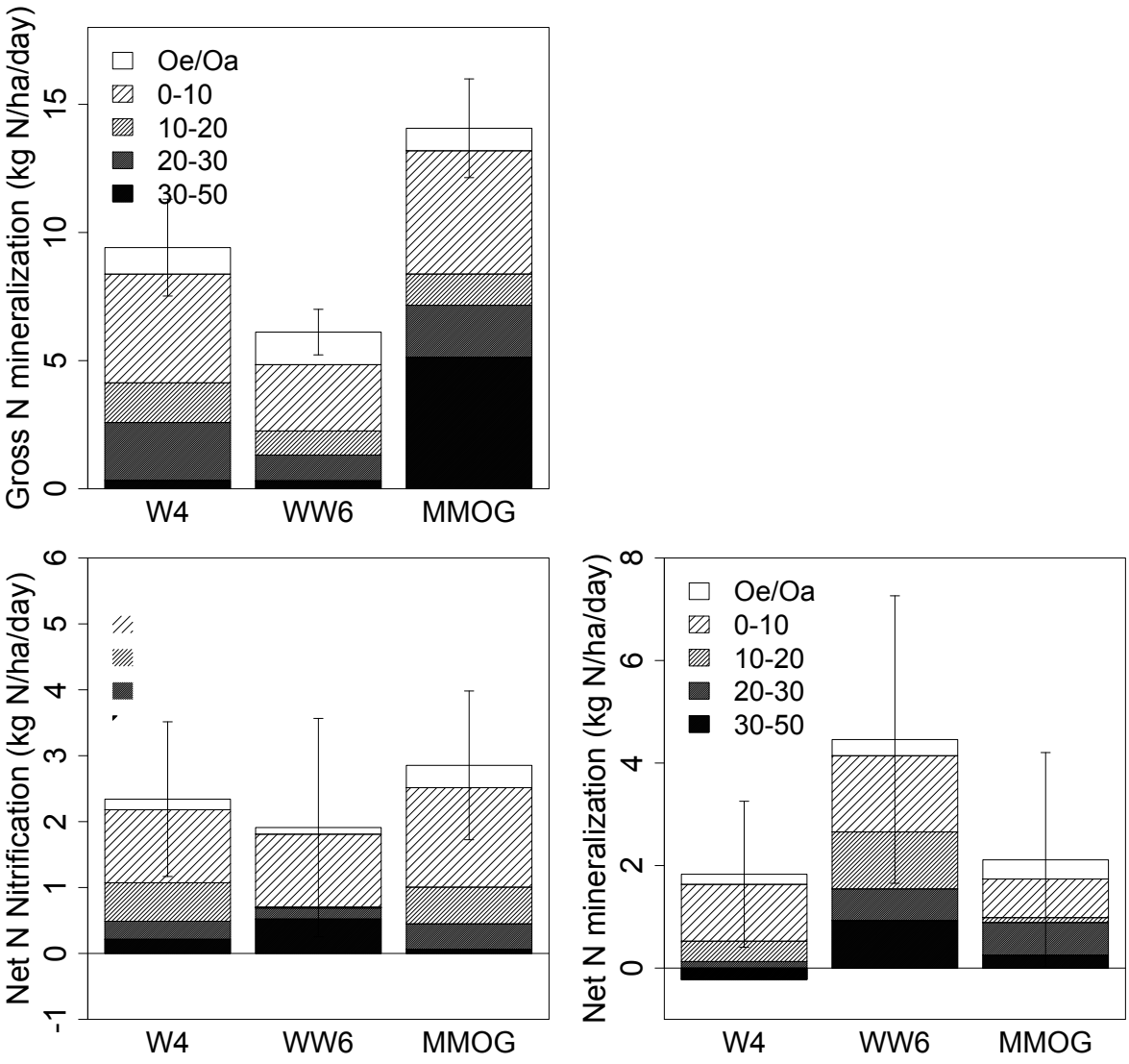

Figure 4. Daily gross and net N cycling rates per unit area for the three sites. Bars are the total mean values and are divided by soil depth. Error bars represent standard errors of the total profile mean

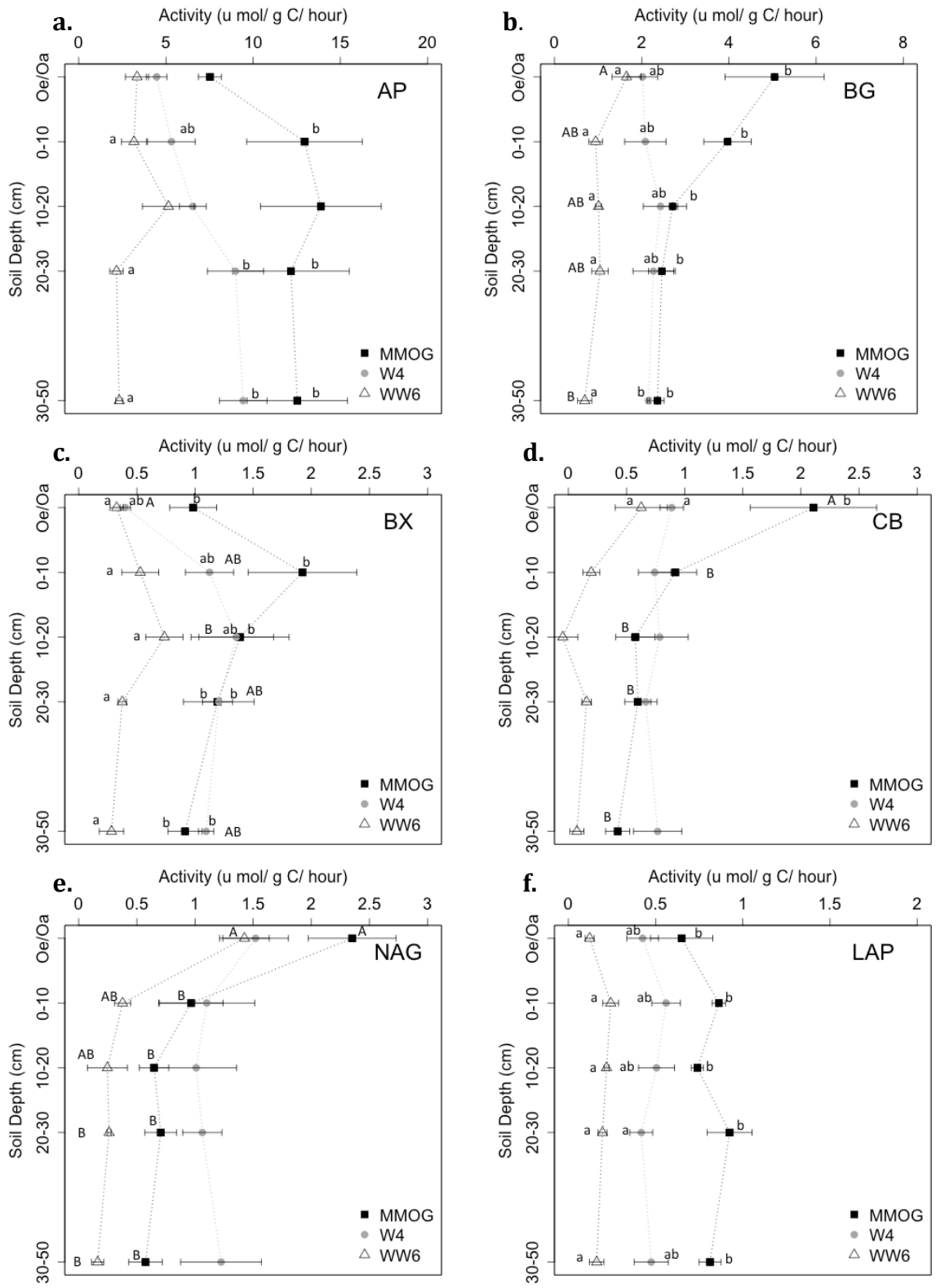

Figure 5. Potential enzyme activity (umol gC^{-1} h^{-1}) for the six enzymes with depth for the three sites. Different letters denote significant differences. Upper-case letters denote significant differences between depths within a site. Lower-case letters denote differences across sites within a depth. The absence of letters indicates non-significant relationships. Error bars represent standard errors of the mean.

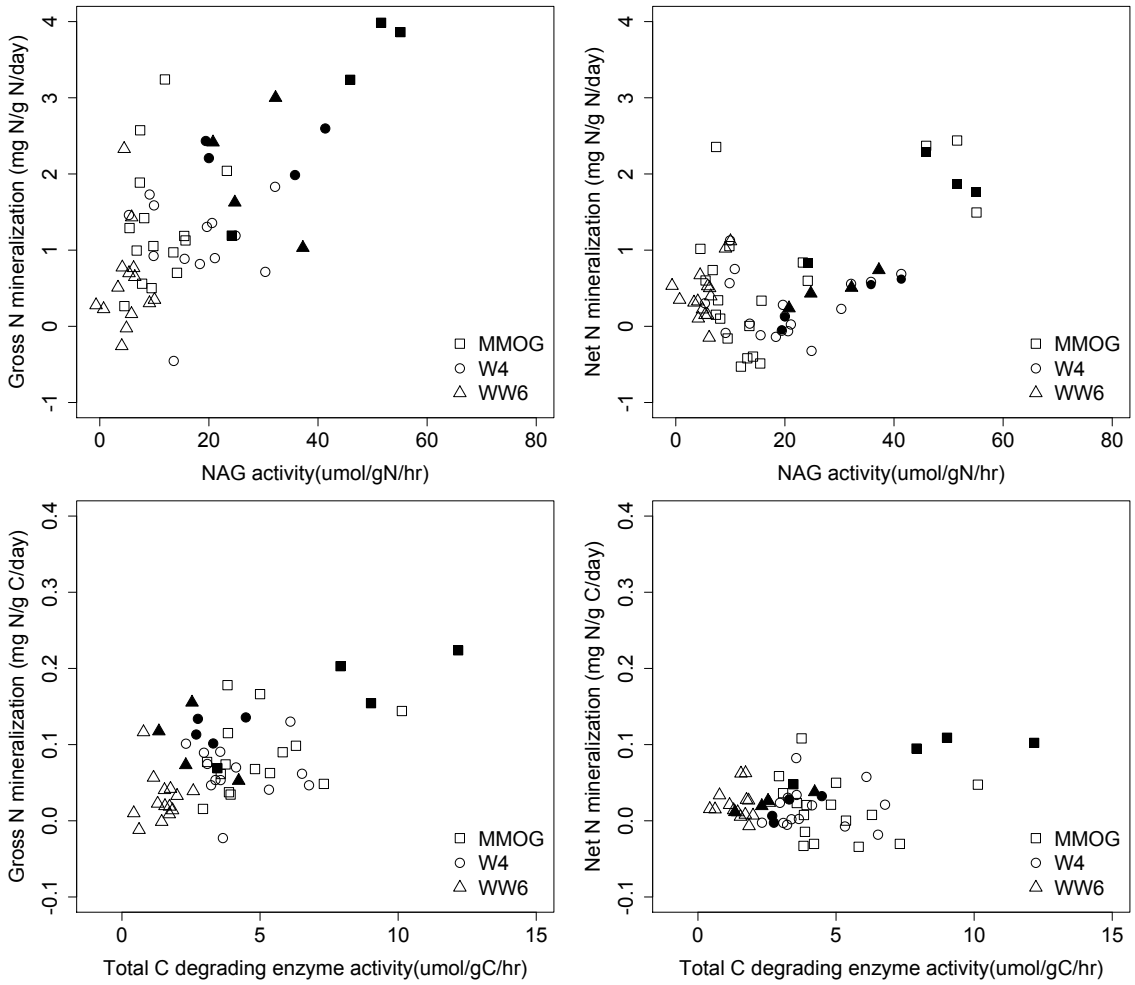

Figure 6. Relationships between gross and net N mineralization and NAG or total C-degrading enzyme activity (reported activity per g soil N). Filled symbols are organic soils, open symbols are mineral soils.

	W4	WW6	Mt. Moosilauke (MMOG)
Latitude / Longitude	N 43.95442° W 71.73004°	N 43.94960° W 71.74051°	N 44.00077° W 71.85913°
Elevation (m)	567.84	635.20	823.57
Approximate stand age (years)	40	100	> 200
Acer saccharum	16%	73%	72%
Fraxinus Americana	24%	0%	0%
Betula allegheniensis	55%	17%	27%
Fagus grandifolia	3%	10%	0%
Other species	2%	1%	1%

Table 1. Location, site age and characteristics at soil sampling sites

Enzyme	Function	Laboratory substrate
Acid Phosphatase (AP)	Releases phosphate groups	4-MUB-phosphate
β-glucosidase (BG)	Releases glucose from cellulose	4-MUB-b-D-glucopyranoside
Cellobiohydrolase (CB)	Releases glucose from cellulose	4-MUB-b-D-cellobioside
β-Xylosidase (BX)	Releases xylose from hemicellulose	4-MUB-b-D-xylopyranoside
N-acetyl glucosaminidase (NAG)	Releases N-acetyl glucosamine group from chitin and peptidoglycan	4-MUB-N-acetyl-b-D-glucosaminide
Leucine aminopeptidase (LAP)	Releases amino acids from protein	L-Leucine-7-amido-4-methylcoumarin hydrochloride

Table 2. Summary of extracellular enzyme functions and substrates used in laboratory assays.

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