# Long-term Warming and Nitrogen Deposition Drive Changes in Enzyme Activity

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# Long-term Warming and Nitrogen Deposition Drive Changes in Enzyme Activity

## 1.1 Abstract

Warming and nitrogen deposition change the earth's ecosystems drastically. Carbon (C) and Nitrogen (N) are two prominent regulators of these ecosystems. Warming causes carbon to be released into the atmosphere as carbon dioxide, a potent greenhouse gas. It also results in increased deposition of N, the most limiting nutrient for terrestrial ecosystems, in soils. Microbial extracellular enzyme activity (EEA) controls how carbon and nitrogen flow through the soil ecosystem and recent work has shown that enzymes are sensitive to environmental change. Enzymes catalyze decomposition in the environment and such changes may alter soil carbon cycles. This study is a small-scale simulation of what the earth's climate could be like 50 years from now. Every enzyme plays a different role in environmental cycling. All four enzymes we tested varied differently when subject to environmental factors such as warming, nitrogen, and warming and nitrogen combined. Separately, nitrogen deposition and warming caused activity in some enzymes to increase and suppressed activity in others, but together nitrogen and warming had an antagonistic effect. We now can better understand how effectively our environment is able to regulate itself. When once factor, such as temperature, increases another one, like N, increases in response thus mitigating the result of one or the other on enzyme activity. Our data will be used to expand our knowledge on the effects of long-term warming on EEA in soil.

## 1.2 Introduction

Earth's atmosphere is projected to warm between 2 °C to 6 °C as a result of anthropogenic enterprises such as mining, burning fossil fuels, widespread urbanization, and industrialization, and increase pesticide use (Riebeek, et al., 2010). These activities release greenhouse gases, like carbon dioxide, methane, nitrous oxide, water vapor, and ozone into the atmosphere at an increased rate. Such environmental alterations have caused, and will continue to cause, significant disruption to all ecosystems as the increase

in temperature is projected to warm soils, which will increase respiration and decomposition as well as atmospheric nitrogen deposition induces the outflux of carbon into the atmosphere, furthering climate change in a positive feedback loop.

Carbon is an important component in the environment giving soils a structure that is advantageous for improving water retention and flow (Schwartz et al.2014). Additionally, soil carbon increases ecosystem productivity by creating a diverse habitat for microbes and aiding in plant growth. Soil carbon also increases soil fertility providing essential nutrients, found in the chemical bonds of soil organic matter, for plant and microbial growth.

Microbes play an integral role in the soil carbon cycle. They decompose soil organic matter (SOM), making the nutrients contained within SOM readily available for plant use. Additionally, microbes work to develop a layered soil structure. These two actions are paramount to the success of all plant growth as well as carbon and nitrogen cycling. As soils warm, both microbe activity, through enzyme production, and the rate of EEA, have been shown to increase in the short term (Wallenstein et al., 2009) will likely result in the rate of nitrogen and carbon cycling to escalate leading to further warming of earth's entire ecosystem through increased carbon dioxide ($CO_2$). In the long-term, as warming increases, EEA will decline (Allison et al., 2010b) and as substrate availability is reduced in response to a reduction in soil moisture and increased N-deposition, the rate of plant growth will decline as well.

Nutrients in SOM are accessed through enzymatic breakdown of carbon structures. Extracellular enzymes are macromolecules that are created within microbial cells. They are secreted into the environment acting as catalysts in the decomposition of complex polymers. By secreting enzymes, microbes are able to initiate the breakdown of organic matter which they then extract energy and nutrients from. Enzymes therefore serve to simultaneously aid microbes in obtaining resources and initiating nutrient cycling. Soil enzymes increase the rate of plant matter decomposition releasing nutrients that then become available for plant use. When plant residues decompose, they release $CO_2$ into the atmosphere. Living plants take in $CO_2$ depositing it in SOM creating carbon sinks. Increases in plant C inputs to the soil increase C sequestration and is one mechanism for

increasing SOM. Plant growth requires available soil nutrients to be efficient, and nutrients can become available through enzymatic decomposition of SOM thus, the carbon cycle and plant growth hinges on enzyme activity in soils to free nutrients and release $CO_2$.

Short-term experimental research has shown enzyme activity to respond negatively to warming (Morrison et al.2011). Fungal abundance in soil often declines as a result of long-term warming (Morrison et al.2011). It is likely that enzyme activity will decrease with warming as well. This correlation is apparent when we look at fungi, plant roots, and bacteria, some of the prime producers of enzymes in the environment. Fungi produce different enzymes than some bacteria in the environment. They are the major producers of enzymes which decompose lignin in the environment. Therefore, they are critical to nutrient cycling and decomposition in the natural ecosystem. If fungal abundance is decreased with warming, so will enzyme abundance, leading to a reduction in overall activity.

In addition to warming, nitrogen deposition is another factor that has been seen to modify, regulate, or repress enzyme activity (Whalen et al. 2018). N deposition slows leaf litter and lignin decomposition (Whalen et al. 2018). The increased N in soils changes the activity and composition of microbial communities. The result is that enzyme production is suppressed thus reducing leaf litter and lignin decomposition.

As soil temperature increases, it is likely that EEA will initially increase as well. After a sustained period of warming, side effects of these higher temperatures, such as N deposition and moisture loss, EEA will be suppressed. Because enzymes help to regulate cycling in the environment, if their activity is inhibited by warming it is likely that the rate of decomposition will decline. Decomposition is paramount to maintaining a healthy layer of SOM. With lower rates of decomposition, SOM quality will plummet and therefore, so will plant growth as plants require nutrients that only become available when decomposition occurs. Without plants present to maintain soil stability and structure, emissions of C will increase adding to the already heightened pool of C in the atmosphere resulting from decreased plant growth and thus a declining rate of C

sequestration and storage. Furthermore, this will detrimentally impact soil health furthering the negative cycle.

## 1.3 Statement of Purpose

In 2013 soil warming and nitrogen deposition were implemented at the Harvard forest to simulate what the earth could be like about 50 years from now. The objective of this study is to examine the effects of long-term warming and nitrogen deposition on EEA in soil. We hypothesize that EEA will decrease due to simultaneous soil warming and nitrogen deposition. The increase in EEA, as a result of initial warming, will eventually be suppressed as N addition has been seen to act as a limiting factor in the environment. The rate of plant growth will also decline in response to warming which will result in a reduction of available decomposable SOM. We will address my objective by analyzing soils heated 5°C above ambient soil temperature and adding nitrogen to soils for 13 years.

## 1.4 Methods

### 1.4.1 Site description:

This research was done at the Prospect Hill Tract of the Harvard Forest Long Term Ecological Research Site in the Petersham, Massachusetts, USA. The Soil Warming and Nitrogen Addition (SWaN) site is made up of even-aged, mixed hardwoods, including red oak, black oak, red maple, striped maple, American beech, white birch, and American chestnut. The soils in the region are a fine, loamy, and mixed mesic. The annual temperature is 7°C reaching a high of 32°C during the summer months and a low of -25°C during the winter (Contosta et al. 2011). Average total precipitation is 1100 mm (Boose et al. 2002).

At the SWaN site, soil plots have experienced a full factorial treatment structure of +5°C warming, + 50 kg N $ha^{-1}$ $year^{-1}$, or a mix of both simultaneously, above ambient soil conditions for 13 years. This was accomplished using buried heating cables placed at a 10 cm depth below the soil surface and spaced 20 cm apart (Contosta et al.2011). The cables were extended 10 cm further than the perimeter of each individual plot in order to

decrease possible heat loss. Cables were not buried in unheated control locations. Adjacent controls from, part of a different experiment, showed no variation in C and N fluxes suggesting that disturbance had no lasting effects on soil processes (Peterjohn et al. 1994). Nitrogen was added to the plots in the form of liquid ammonium nitrate.

### 1.4.2 Sampling design:

The SWaN experiment is made up of 23 plots of which 6 are controls, 5 are heated, 6 are nitrogen addition and 6 are heated with nitrogen. The plots each represents an experimental unit. Two subsamples were taken from each plot. In total, 138 samples were taken. The organic horizons were all sampled up until the mineral soils in a 10 cm x 10 cm area. The depth of the organic horizon was measured to the nearest mm. The mineral horizon was then sampled to a depth of 20 cm using a Giddings slide hammer. Starting at the surface of the mineral horizon in the location the O horizon was taken, soil samples were removed in 10 cm increments. Once the sampling cylinder reached the first 10 cm increment (measured and marked on the cylinder), the soil core was be removed from the sample hole. The process was repeated one more time to reach the 20 cm depth point. Both the organic and the mineral soil horizon depths were removed from the cylinder and an initial mass of soil was taken. The organic horizon and mineral soil samples and stored in a plastic bags that were placed on blue ice. The samples were then transported back to the lab covered with damp paper towels in order to maintain high humidity. They were then placed in a 4°C fridge.

Soils were homogenized and their initial masses were taken. To determine the water moisture content, soils were dried, at 60°C for the organic horizon and 105°C for mineral soils, until a constant mass was gained. Their mass was then recorded again. Soils were then sieved through a 2 mm sieve into conical tubes, each between 5 and 10 g, and stored at -80°C.

### 1.4.3 Soil enzyme analyses:

EEA of organic soils of Cellobiohydrolase (CBH), 4-nitrophenyl-β-D-glucopyranoside (BG) and 4-nitrophenyl-N-acetyl-β-D-glucopyranoside (NAG), and

Acid Phosphatase (PHOS) was determined using a fluorometric enzyme assay. Briefly, pulverized soil and litter slurries of each subsample were made by blending 0.5-1 g of the subsample with 60 mL of sodium acetate buffer, adding an extra 65mL buffer to rinse the blender. Sample slurries were then transferred to a 500mL beaker, which were placed on a stir plate. 200 μL of each soil slurry was then pipetted into a 96 – well plate using a wide-mouthed pipette. Each sample was assayed to then determine numerical enzyme activity. These assays were each replicated a second time to decrease potential error.

Using the data from the assays, which gave use EEA values in relative fluorescence units (RFU) or net fluorescence (NF), the total final activity of all four enzymes (CBH, BG, PHOS, and NAG) was then calculated. First, we determined the Quench Coefficient using the formula:

$$\frac{(Quench\ RFU\ -Sample\ Control\ RFU)}{Standard\ RFU}$$

where the "quench" relative fluorescence units or RFUs are fluorescence measurements of standard slurry + sample slurry. We then calculated the Emission coefficient, or EmC, using the formula:

$$\frac{Standard\ RFU}{nmoles\ standard\ added\ to\ wells}$$

Next, we calculated the net fluorescence (NF) for both of our samples, replicate A and replicate B for each sample, using the formula,

$$\left(\frac{Sample\ Assay\ RFU - Sample\ Control\ RFU}{QC}\right) - Substrate\ Control\ RFU$$

We then calculated the final activity of both of our samples separately, using the equation,

$$\frac{NF\ x\ 125\ ml}{EmC\ x\ 0.2\ ml\ x\ incubation\ time\ (h)\ x\ soil\ mass\ (g)}$$

With these final activity values, we were then able to calculate the standard errors and means for both samples individually. Final activity is expressed as μmols/g*hr.

$$(\frac{NF1 * 125}{EMC}) * 0.2ml * 0.5nmoles * Sample\ mass$$

## 1.5 Results

We assayed four enzymes, CBH, BG, NAG, and PHOS to determine activity comparing standard errors to analyze our values (Table 1).

*Table 1: The table represents all enzymes, their activity among the different treatments in μmols/g*hr and the standard error was calculated for each mean activity value.*

| Treatment | CBH | | | BG | | | NAG | | | PHOS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 0.65 | ± | 0.34 | 0.92 | ± | 0.25 | 1.27 | ± | 0.46 | 0.76 | ± | 0.32 |
| Heated | 0.53 | ± | 0.39 | 1.40 | ± | 0.51 | 1.21 | ± | 0.65 | 1.96 | ± | 0.44 |
| Heated x Nitrogen | 0.56 | ± | 0.31 | 1.08 | ± | 0.41 | 1.28 | ± | 0.40 | 1.11 | ± | 0.37 |
| Nitrogen | 0.29 | ± | 0.31 | 0.43 | ± | 0.29 | 1.13 | ± | 0.38 | 0.86 | ± | 0.48 |

Overall, across the entire study, NAG displayed the greatest activity at 1.22 μmols/g*hr with a standard deviation (SD) of +/- 0.47 μmols/g*hr (Table 2). The activity of the enzyme PHOS, closely followed at 1.17 μmols/g*hr with its own standard error being +/- 0.40 μmols/g*hr (Table 2).

*Table 2: The table shows average enzyme activity for all enzymes in μmols/g*hr. This encompasses all treatments and the control for each enzyme.*

| Enzyme | Mean | SE |
|---|---|---|
| CBH | 0.51 | 0.14 |
| BG | 0.96 | 0.15 |
| NAG | 1.22 | 0.19 |
| PHOS | 1.17 | 0.17 |

The margin between BG and PHOS was greater than the margin between NAG and PHOS being that BG showed an average rate of 0.96 μmols/g*hr activity, about a .21 μmols/g*hr activity margin. The average standard deviation of BG for the whole study was +/- 0.37μmols/g*hr. At 0.51 μmols/g*hr, CBH showed the least activity and also had

the smallest average standard deviation at 0.34µmols/g*hr. (Figure 1). Because variability was only determined to be significant if error bars did not overlap, we can see that CBH was the only enzyme that displayed notable variability overall (Figure 1). The error bars for all of the other enzymes overlapped, but the error bars for CBH did not.

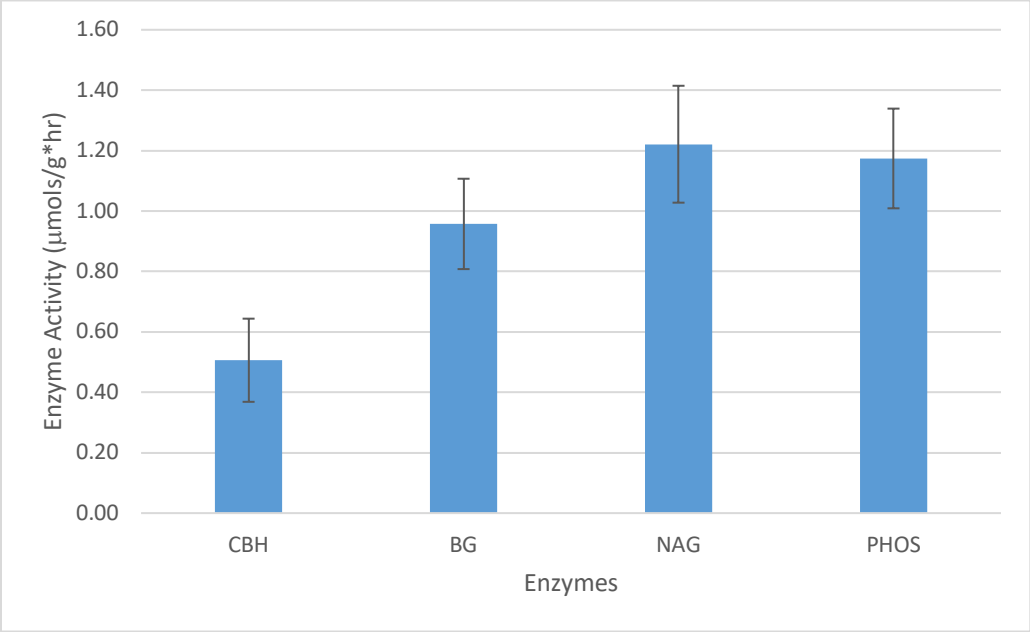


*Figure 1: This figure shows the average of the activity of all four enzymes in µmols/g*hr.*

For all four enzymes, activity was quite variable in response to warming, nitrogen deposition, and the combination of warming and nitrogen deposition. Warming caused both BG (Figure 2, a.) and PHOS (Figure 2, d.) activity to increase, in comparison to their controls. PHOS did display greater variability in this heated treatment as we saw that its error bar for this treatment did not overlap with any of the others. CBH (Figure 2, a.) and NAG (Figure 2, c.) activities were seen to decrease with warming, compared to their controls. CBH activity decreased about 0.13 µmols/g*hr from 0.65 µmols/g*hr to 0.52 µmols/g*hr while NAG activity decreased very minimally, only about 0.05 µmols/g*hr from 1.25 µmols/g*hr to 1.20 µmols/g*hr.

The overall activity in the plots subjected to nitrogen deposition was the least for CBH, NAG, and BG across all treatments although, BG did show some variability (Figure 2, b., and c.). Activity of PHOS in the nitrogen plot was much lower than the heated treatment, but slightly higher than its control (Figure 2, d.).

The treatment of HxN resulted in EEA both increasing and decreasing in comparison to their controls. When exposed to both heat and nitrogen deposition simultaneously, CBH activity was reduced by 0.09 µmols/g*hr, in relation to its control, from 0.65 µmols/g*hr to 0.56 µmols/g*hr (Figure 2, a.). CBH activity in the heated plot was greater than the HxN treatment, increasing from 0.53 µmols/g*hr to 0.56 µmols/g*hr (Figure 2, a.). Both BG and PHOS activity increased compared to their controls but their activity was suppressed when compared to their heated counterparts (Figure 2, a. and d.). The activity of NAG, in the HxN treatment, was greater than in its heated treatment. Compared to its control, NAG displayed almost no variation increasing only a fraction from1.27 µmols/g*hr to1.28 µmols/g*hr (Figure 2, c.).

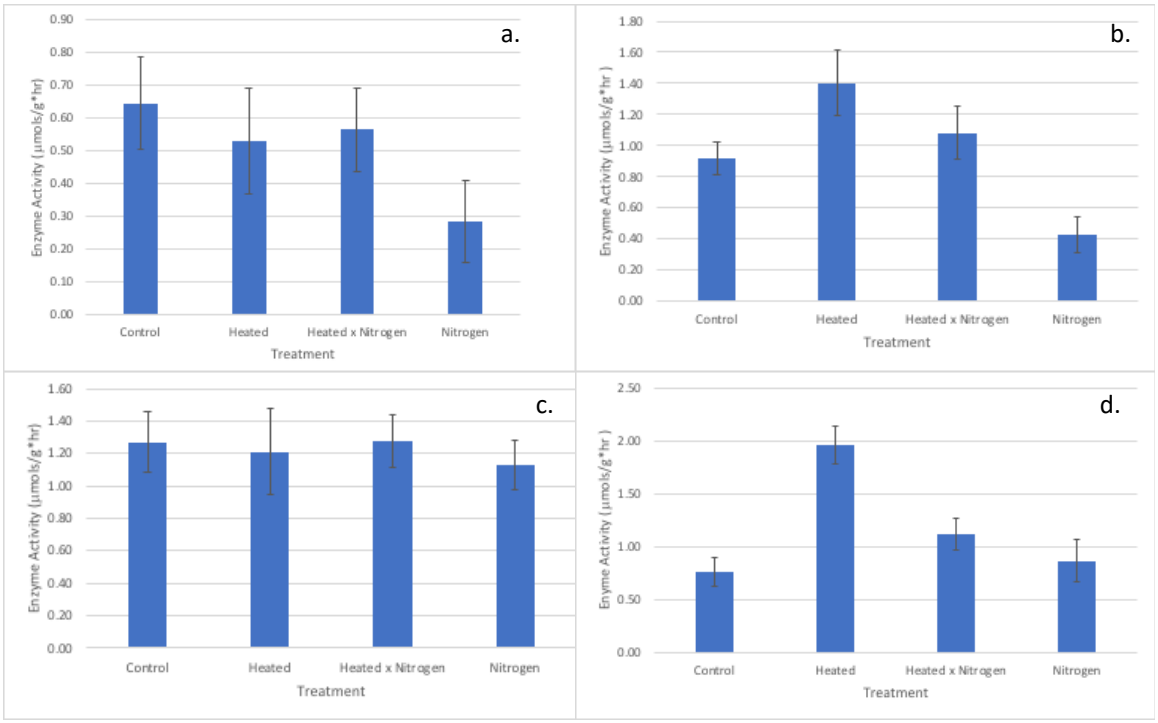


*Figure 2: This figure shows CBH, BG, NAG, and PHOS activity in µmols/g*hr. The error bars represent the standard error in the data. Significance in variability is determined if the bars do not overlap.*

## 1.6 Discussion

We examined the responses of CBH, BG, NAG, and PHOS to warming at +5 °C above ambient temperature, nitrogen deposition at + 50 kg N $ha^{-1}$ $year^{-1}$, and the

combination of warming and nitrogen together. The trends we saw varied greatly across all treatments, however the HxN treatment seemed to mitigate the result N deposition or heat had on EEA alone. Overall, NAG displayed the greatest substrate consumption on average and across every treatment (Figure 2, c.). Furthermore, NAG responded very little to environmental change. NAG activity increased by 0.06 μmols/g*hr when heated and by 0.01μmols/g*hr in its HxN plot and decreased by and 0.14 μmols/g*hr when just subject to nitrogen deposition. The data calculated for NAG didn't display any significant variability. The fact that NAG was significantly resistant to environmental changes may have something to do with the fact that NAG is a very strong enzyme degrading some of the most persistent compounds in the environment. It is possible that heat and N are not enough to change its ability to decompose

We saw a clear correlation between greater average activity and higher standard error. On average, NAG was noted for displaying the highest rate of activity. It can also be said that NAG had the largest average standard deviation. PHOS had a lower average rate of activity than BG and CBH and its standard deviation was higher than theirs, but lower than the SD of NAG as well. It was the same with BG and CBH. BG displayed a greater rate of activity than CBH, overall, and its SD was greater as well.

The average activity of CBH was less than half the value of NAG (Table 1). BG activity was almost double the activity of CBH. PHOS activity was greater than BG and CBH at only 0.05 μmols/g*hr bellow NAG activity. Overall, BG responded the most to all three treatments, increasing and decreasing activity to the largest extent in treated soil (Figure 2, b.). Low PHOS activity could mean that the system is not in demand of P, which still indicates that N is the limiting nutrient in these systems.

Fungi, especially arbuscular mycorrhiza fungi (AMF) and saprotrophic fungi, prime producers of NAG, are greatly influence nutrient cycling in the soil ecosystem. It seems likely that the rate of enzyme activity may have had a lot to do with root and mycelial presence in addition to the warming, nitrogen deposition and, HxN combined. (Nottingham et, al. 2013) showed that microbial communities and organic nutrient cycling are both influenced by root and AMF mycelia availability. C input in soils occurs through plant sequestration and fine root exudation and turnover. Additionally, AMF

mycelia provides C, derived from plants, to the wider microbial community. Because fungal abundance has been seen to decline with warming (Morrison et al. 2011), then the activity of NAG and CBH decreasing with warming correlates to this slowed fungal growth. Unpublished data from the Frey lab suggests that mycorrhizal colonization rates in the same soils is decreased under warming and nitrogen addition alone but is significantly increased when soils are warmed and fertilized simultaneously.

Furthermore, BG and CBH degrade cellulose in the environment (Ljungdahl & Eriksson 1985). NAG contributes to the degradation of chitin (Sinsabaugh et, al. 2005) and PHOS extracts phosphates from soil proteins making them available for plant use. That said, EEA is greatly affected by soil N, C, and P availability and ratio as well as soil pH (Sinsabaugh et, al. 2008). Excess or suppression of these elements greatly influences enzyme activity and their abundance in soils is largely affected warming and/or nitrogen deposition. This means that, alongside warming, nitrogen deposition, or HxN respectively, soil C, P, and the pH of the soil may also have been causing the variation in enzyme activity.

Specifically, competition for phosphate is a factor that has been seen to constrain microbial abundance, and therefore enzyme abundance as well, in the presence of AMF (Nottingham et, al. 2013). This occurs when soil is a highly saturated or has a pH value bellow 5.5 or between 7.5 and 8.5, something that directly correlates to N abundance. P-availability is limited by these factors as it is released much more slowly into soil under such conditions (Soil Quality). As a result, phosphate is formed less rapidly, and enzymes have more difficulty finding it in SOM.

In support of our hypothesis, nitrogen deposition seemed to reduce enzyme activity to the largest degree for CBH, NAG, and PHOS. As was noted in a recent study, N deposition slows leaf litter and lignin decomposition, which is the direct result of decreased enzyme activity (Whalen 2018). This may be explained by the fact that, nitrogen actually suppresses enzyme activity in lignin decay (Carreiro et al., 2000; DeForest et al., 2004) because lignin protects the decay of cell- wall polysaccharides, the cellulose, a C co-substrate that helps initiate lignin degradation, and litter N reduces the ability for N to limit C use by decomposers (Talbot et al., 2012).

CBH and NAG activity were both reduced with warming, which is consistent with recent findings stating that long-term warming would reduce enzyme activity (Allison et al., 2010b). Contrary to expectations, both BG and PHOS actually increased when heated. This could be a result of the fact that, under warming, there could be a greater demand for simple carbohydrates, which are important nutrients for BG. Additionally, an increase in PHOS suggests that the ecosystem being studied has an increased demand for phosphorous. These findings are concurrent with a study relating that, in the short term, microbe activity, through the rate of soil enzyme activity, increases with warming (Wallenstein et al., 2009).

The compilation of HxN in soils documented a wide range of variation. When looking at the activity of all enzymes in HxN treatments, compared to their heated treatments, we can see clearly that, somehow depositing N in the soil along with increasing the temperature seemed to mitigate the disruption, either negatively or positively, in order to maintain a rate of activity more similar to its control (Figure 2, A, B, C, D). The same is the case when compared to the N treatments for all enzymes; HxN seemed to follow that middle line closest to the control and in between the rates calculated in the soil with N-abundance or the heated soil. It seems that if heat and N deposition either reduce or stimulate enzyme activity, combining the two offsets the full capacity of that response.

## 1.7 Conclusion

Soil warming and N deposition were shown to alter enzyme activity and that the combination of HxN mitigates the variance caused by N and soil warming. Every enzyme we tested, CBH, BG, NAG, and PHOS responded differently to each environmental variation. We hypothesized that EEA would be negatively affected by both warming and N deposition. Overall, our hypothesis was not completely supported, because only some of the enzymes CBH, BG, NAG, and PHOS responded as anticipated. A change in EEA likely reflects the organisms that showed the greatest variation, this more widely being determined by the SOM present in the particular plot. Due to the fact that each enzyme plays a different role in nutrient cycling in the broader ecosystem, their ability to

withstand environmental changes varied greatly. Overall, EEA did vary with warming and N deposition although, across the board, the results were inconsistent. While warming caused EEA to increase for some enzymes it suppressed activity in others and similarly, whilst certain EEA declined with N-deposition, others showed heightened activity.

Altogether, HxN together had the most unanticipated outcome. The two factors seemed to have an antagonistic effect on EEA. Therefore, the activity of the samples in this HxN site displayed rates that were in between samples from just N deposition sites and the sites where only warming took place. The EEA did not return to the control levels, but it was numerically closer than the rates in the other treatment plots.

This HxN treatment best displays the natural ecosystem in 50 years. There is a correlation between warming and increased N deposition in the environment meaning that it is unlikely that a region will naturally experience warming without N deposition as well. Although it seemed as though the rate of enzyme activity would increase/decrease drastically, due to warming or N deposition, causing either a buildup or increased breakdown of OM, and an excess release of C into the atmosphere, we can now be certain that the results will not be quite as detrimental as anticipated. This is due to our newfound knowledge that the effect on EEA of warming and nitrogen together mitigates the result of warming or N separately.

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