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Influence of Decreasing Temperature on Soil Microbial Activity in a Boreal Shield Ecozone

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Abstract: Although the focus on warming is imperative to address climate change, addressing the effects of cooling is arguably just as relevant in characterizing the enzyme response to temperature fluctuations. In this study, the response of enzyme activity to diminishing temperatures in the field was evaluated. The targeted sites were located in the City of Greater Sudbury in Northern Ontario, Canada. Microbial activity variations during the summer and fall seasons were measured using freshly collected soil samples. The soil was acidic with an average pH of 4.8 and the level of soil organic matter was 11.3%. The local atmospheric temperatures recorded during the sampling period in Sudbury were 21.3°C (August), 2.3°C (October), and -6.3°C (November), respectively. Overall, nine enzymes were targeted including β-Glucosidase (BG), Cellobiohydrolase (CBH), β-N-Acetylglucosaminidase (NAGase), Aryl Sulfatase (AS), Acid Phosphatase (AP), Alkaline Phosphatase (AlP), Glycine Aminopeptidase (GAP), Leucine Aminopeptidase (LAP) and Peroxidase (PER). Microbial activities in the field during summer and fall seasons varied significantly with different enzymes analyzed. BG, CBH, NA Gase, and AS remained unchanged despite the decreasing temperature. The activities of AP, AlP, and PER increased significantly from the first sampling in August to October 2020. AlP, LAP activity decreased whereas LAP activity increased from October to November. Considering the complexity of the forest ecosystem, some factors other than the temperature might affect microbial activities in field conditions. Data on enzymatic activities in soil samples from different ecological conditions should be interpreted with caution because of the various effects of environmental variations on soil functions.

Keywords: Enzymatic Activities, Microbial Communities, Low Temperature, City of Greater Sudbury

Introduction

Soil enzymes are mostly extracellular and come from a variety of microorganisms that include bacteria, fungi, and archaea (Traving *et al.*, 2015). Enzyme activity is one of the few parameters that determine soil health but the further characterization is needed to understand the effects of different environmental factors such as substrate availability, temperature, and pH (Andersson and Nilsson, 2001). Microorganisms and extracellular enzyme activity have an optimal temperature of operation (Zhang *et al.*, 2018). Lower temperatures cause a decrease in cell membrane fluidity in microorganisms and do not provide the activation energy necessary for extracellular enzymes to react with the substrates (Siliakus *et al.*, 2017). In soil, deviations in enzyme activity from the Arrhenius model which predicts an exponential increase in reaction rate with temperature have prompted researchers to explore different approaches such as the Macromolecular Rate Theory Model which incorporates thermodynamic temperature optima as arising from heat capacity differences between isoenzymes (Alster *et al*., 2016). These deviations from in vitro conditions indicate that the response of soil enzymes to temperature is multifaceted and depend on how temperature interacts with other factors such as nutrient availability, pH, moisture, metal cations, and adsorption to colloids (Alster *et al*., 2016).

Studies involving artificial warming in the winter season *in situ* showed no difference or increases in microbial biomass over time (Ueda *et al.*, 2013). Higher temperatures increase oxidative enzyme activity, yield variable increases in hydrolytic enzyme activity, and increase C, N, and P mineralization and acquisition (Meng *et al*., 2020; Gao *et al*., 2020). Global

temperatures have increased by an average rate of 0.18°C per decade since 1981 and 19 of the 20 highest global temperatures have been recorded since 2001 (NASA/GISS 2020). Although the focus on warming is imperative to address climate change, addressing the effects of cooling is arguably just as important in characterizing the enzyme response to temperature fluctuations. Some researchers proposed that cooling yields a lower affinity and acquirement of substrates even for acclimated microorganisms (Wiebe *et al*., 1992). Pietikainen *et al*. (2005) showed that lower temperatures were associated with a higher fungal to bacteria ratio which affected the number of extracellular enzymes released. Temperature decreases have been correlated with decreased water, nutrient uptake, and photosynthesis in vegetation, subsequently affecting the exchange of nutrients with enzymes (Hajihashemi *et al.*, 2018; Nievola *et al.*, 2017).

The specific objective of the present study was to evaluate variations of microbial activities under decreasing field temperatures.

Materials and Methods

Sampling Site Location and Characterization

The targeted sites were located in the City of Greater Sudbury in Northern Ontario, Canada. The region is built on the foundation of a basin formed by a crater and consists of bedrock and thin soils (Narendrula and Nkongolo, 2015). Soils and lakes in the Sudbury have a high content of heavy metals such as nickel, copper, and arsenic likely as a result of prevalent mining and smelting in the area (Feisthauer *et al.*, 2006; Nkongolo *et al*., 2013). The Kelly Lake site (coordinate: 46°26'42.0"N 81°03'18.0"W) recently underwent a liming remediation program in 2016 using dolomite limestone (Narendrula and Nkongolo, 2017). The map showing the site location is decrypted in Fig. 1. Metal analyses for the region have been described in detail by Nkongolo *et al.* (2013, 2016) and Narendrula and Nkongolo, (2015). The soil pH for the targeted sites was measured using the method reported in Nkongolo *et al.* (2013). Organic matters were determined using the Loss On Ignition (LOI) analysis at Tesmark Inc. (Sudbury).

Soil Collection and Microbial Response to Decreasing Seasonal Temperatures

To measure the variation of enzymatic activities in the field at different times, soil samples were collected from the Kelly Lake site (limed area) at three different times in the summer and fall of 2020. These collection dates at the same locations include August 12, October 17, and November 18. The local atmospheric temperatures recorded during these days in Sudbury were 21.3°C, 2.3°C, and -6.3°C, respectively. Three transects of 10 m diameter were used for soil collection. Ten subsamples pertranset/replicate (three replicates) were collected from the organic layer. The samples were sieved in a 1 mm mesh to remove debris, rocks, and plants and stored at 4°C. The sieved soil samples were analyzed immediately after collection for microbial activities.

Enzyme Analysis

Nine different enzymes were selected for analysis based on their ability for catalyzing reactions and their functions involving geochemical processes (Table 1). They include β-Glucosidase (BG), Cellobiohydrolase (CBH), β-N-acetylglucosaminidase (Nagase), Aryl Sulfatase (AS), Acid Phosphatase (AP), Alkaline Phosphatase (AlP), Glycine Aminopeptidase (GAP), Leucine Aminopeptidase (LAP) and Peroxidase (PER). All enzymes were assayed at their optimal pH values using a 96-well plate reader and the Fluostar optima from BMG Technologies. Assays for β-glucosidase, cellobiohydrolase, β-N-acetylglucosaminidase, arylsulfatase, acid phosphatase, and alkaline phosphatase were conducted using p-Nitrophenol (pNP) linked substrates. Glycine aminopeptidase and leucine aminopeptidase activities were assayed using p-nitroanilide. Peroxidase and polyphenol oxidase activity was assessed using the L-DOPA (L-3,4-dihydroxyphenylalanine) substrate. Substrate concentrations of 5 mm were used for all enzymes, except Cellobiohydrolase (CBH) and β-N-acetylglucosaminidase (NAGase) due to their solubility (2 mm) and cost. Original protocols for all assays are available on the Environment RCN webpage (http://enzymes.nrel.colostate.edu).

Table 1: Enzymes with corresponding substrate and function

Enzymes	Function	Substrate
β-Glucosidase (BG)	Cellulose degradation, carbon cycling	*p*NP β-D-glucopyranoside
Cellobiohydrolase (CBH)	Cellulose degradation and other beta-1,4 glucans, carbon cycling	*p*NP-β-D-cellobioside
β-N-Acetylglucosaminidase aka chitinase (NAGase)	Chitin degradation, carbon/nitrogen cycling	*p*NP-N-acetyl-β-D-glucosaminide
Arylsulfatase (AS)	Produces plant-available sulfates, sulfur cycling	*p*NP sulfate
Acid Phosphatase (AP)	Produces plant-available phosphates, phosphorus cycling	*p*NP phosphate (buffer pH 5.0)
Alkaline Phosphatase (AlP)	Releases ester bound phosphates, phosphorous cycling	*p*NP phosphate (buffer pH 9.0)
Glycine Aminopeptidase (GAP)	Degrades protein into amino acids, nitrogen cycling	Glycine-*p*-nitroanilide
Leucine Aminopeptidase (LAP)	Degrades leucine and other hydrophobic amino acids from protein, nitrogen cycling	L-Leucine-*p*-nitroanilide
Peroxidase (PER)	Lignin and tannin (polyphenols) degradation, carbon cycling	L-3,4-dihydroxyphenylalanine (DOPA)

pNP: 4-nitrophenyl

Fig. 1: Location of the sampling site within the Greater Sudbury Region. Site: Kelly Lake (coordinate: 46°26'42.0"N 81°03'18.0"W). *Represent smelter sites

In preparation for the enzyme activity analysis, four grams of soil were added to forty milliliters of 50 mm sodium acetate buffer (pH 5.0) and vortexed at a high speed for 2 min to create the homogenate. Aliquots of 200 µL of the homogenate were added to polypropylene tubes that contained 200 µL of the substrates. Since the peroxidase substrate was used to analyze the peroxidase and the polyphenol oxidase activity, only the tubes which analyzed the peroxidase enzyme activity had 10 µL of 0.3% H_2O_2 (hydrogen peroxide) added before incubation. Other substrates except peroxidase were mixed well before being placed on a rotary shaker for 2 h at room temperature. The peroxidase tubes were placed on a spinning wheel for 2 h at 4°C. Once the incubation was completed, the tubes containing the substrates and the homogenate mixture were centrifuged at 3200 × g for 4 min and aliquots (100 µL) of the supernatant were taken from each tube and transferred into the microplate. For pNP and p-nitroanilide substrates, 5 µL of 1.0 M NaOH (sodium hydroxide) was added to the wells to stop the reaction. Microplates were read at 405 nm for p-nitro phenol and p-nitroanilide. The microplate was read at 450 nm for peroxidase substrates. Substrate and control samples were used and all assays were performed in triplicates. The absorbance of the assay was corrected by subtracting the combined absorption results for the sample and substrate controls. Enzyme activity was expressed as nmol h^{-1} g $soil^{-1}$. Original protocols for all assays are available on the Environment RCN webpage (https://enzymes.nrel.colostate.edu/enzymes-experimental-protocols.html).

Statistical Analysis

Statistical analyses were conducted using SPSS version 20 for windows (IBM, NY, USA). A Shapiro-Wilk test was used to test for the normality of the enzyme activity data. Paired T-tests were used to determine significant differences in enzymatic activities between successive dates. For any non-parametric data within a given pair, a Wilcoxon test was used to determine significant differences between dates.

Results and Discussion

Changes in Enzymatic Activity as Field Temperature Decreased

The soil was acidic with an average pH of 4.8 and the level of soil organic matter was 11.3%. The temperature during the first sampling (August 12, 2020) was 21.3°C. It decreased significantly during the second sampling date (October 20, 2020) with a mean of 2.3 °C and the third sampling (November 18, 2020) reaching -6.3°C during the sampling day. Six days of freeze-thaw cycles were observed between the second and third sampling where the temperature climbed to 15°C for one day before dropping below -11°C a couple of days later.

Variations of enzymatic activities are described in Fig. 2 to 10. Overall, BG, CBH, NA Gase, and AS activities remained unchanged under decreasing temperature conditions, suggesting that these enzymes were not significantly affected by any environmental changes. AP, AlP, GAP, and PER changed significantly with decreasing temperatures, demonstrating that temperature largely affected their activity. AlP and LAP activities decreased whereas LAP activity increased from October to November. In temperate forests, Baldrian *et al.* (2013) showed that activities for BG and CBH were unchanged from summer to winter whereas NA Gase changed. When considering seasonal temperatures, studies have proposed that soil moisture content was a large confounding variable that affected enzyme activity, especially in areas impacted by drought (Baldrian *et al.*, 2013; Niemi *et al.*, 2007). However, this was likely not a large influencing factor compared to the other studies because the site was by a lake and there were no drastic deviations in typical precipitation. Freeze-thaw cycles common in Northern Climates could have also contributed to the decline of activities for AlP and PER. Freeze-thaw cycles cause an initial increase in C and N mineralization after the first cycle but these parameters including biomass decrease after consecutive cycles (Sorensen *et al.*, 2018). The area sampled during the present study underwent six days of freeze-thaw cycles up to the date of sampling. Abellan *et al.* (2011) demonstrated that soil sampled during winter exhibited higher BG and phosphatase activity than soil sampled from the spring. Although BG was unaffected by seasonal variations, AP and AlP changed with seasonal temperatures. The study also reported significantly higher BG and phosphatase activity at -20°C compared to 4°C, suggesting the presence of intracellular enzymes because of cell lysis (Abellan *et al.*, 2011).

Shifts in the microbial community may influence changes in enzyme activity behavior between seasonal temperatures. In temperate ecosystems, Zhang *et al.* (2014) found an increase in SOC mineralization and C to N ratio in the winter compared to the summer. These increases may explain the stability of enzymes involved in C mineralization such as BG, CBH, and NA Gase and alterations in enzymes involved in N mineralization such as LAP and GAP. Zhang *et al.* (2014) also observed increased fungal to bacterial ratio and gram-negative to the gram-positive ratio in temperate regions. These increases in biochemical characteristics seem to be ecosystem specific because other studies found no increase in fungi to bacteria ratio and decrease in SOC for other ecosystems with different microbial community compositions (Zhao *et al.*, 2017).

Beta Glucosidase, Cellobiohydrolase, and β-N-Acetylglucosaminidase Activities

BG, CBH, and NA Gase remained unchanged across temperatures, indicating low-temperature sensitivity (Fig. 2-4). In contrast to these results, Koch *et al.* (2007) reported higher temperature sensitivities for BG and NA Gase and lower temperature sensitivities for LAP. The lack of significant difference for BG at different temperatures may result from its prevalence in soil compared to other enzymes (Salaberria *et al.*, 2015). The prevalent nature of BG and CBH and the ubiquitous presence of their substrate gives them more flexibility than other enzymes to adjust to their immediate surroundings, especially in response to different temperature fluctuations (Li *et al.*, 2014). Likewise, NA Gase could also adjust to seasonal changes due to chitin being the second most abundant biomass after cellulose (Salaberria *et al.*, 2015). Some microbial communities involved in cellulose degradation were reported to adapt to seasonal changes by increasing cellulose mineralization and reducing labile carbon uptake (Koranda *et al.*, 2013).

Acid Phosphatase and Alkaline Phosphatase Activities

Acid and alkaline phosphatases have a very similar structure and are separated into classes based on one main parameter-the optimal pH at which it operates. The ratio of acid and alkaline phosphatases is used as a parameter to determine the appropriate pH in soils (Dick *et al.*, 2000). In the present study, both AP and AlP increased from august to October, AP remained unchanged whereas AlP decreased significantly from October to November (Fig. 5 and 6). To support this result, studies found a slight increase in phosphatase activity from the spring to the autumn season and attributed the increase to litterfall as opposed to seasonal temperature (Kang *et al.*, 2009). AP may have maintained enzyme activity better than AlP because the optimal pH of AP was closer to the pH of the soil (Margalef *et al.*, 2017). AP is also ubiquitous in soils whereas AlP is rare, thus its activity comes from a larger enzyme reserve (Sun *et al.*, 2020). Additionally, AP and AlP differ in the ions that inhibit them as AP is inhibited by fewer divalent cation chelating agents that may be present in the soil (Dean, 2002). Temperate forests in the north are usually not

phosphorus limited, therefore there is a decreased demand for phosphorus and consequently, a decrease in enzyme activity compared to other enzymes that may be limited by phosphorus (Deforest *et al.*, 2011).

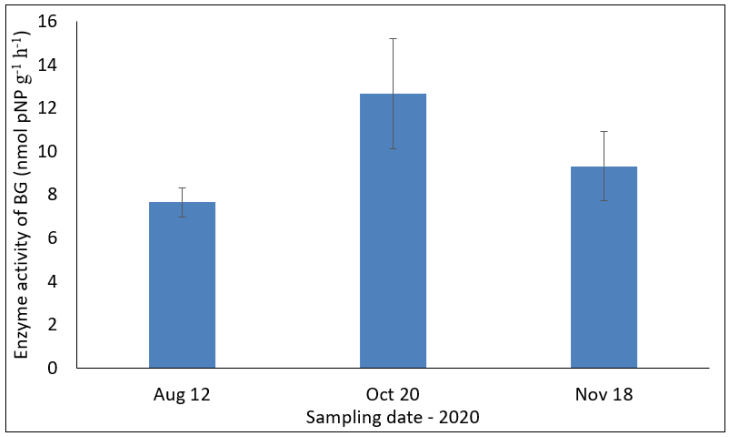

Fig. 2: Activities of β-glucosidase (BG), at different sampling dates. No significant difference was observed between means of BG activities (nmol pNP g-1 h-1) for different consecutive sampling times (dates) based on the paired T-test (P ≥ 0.05).

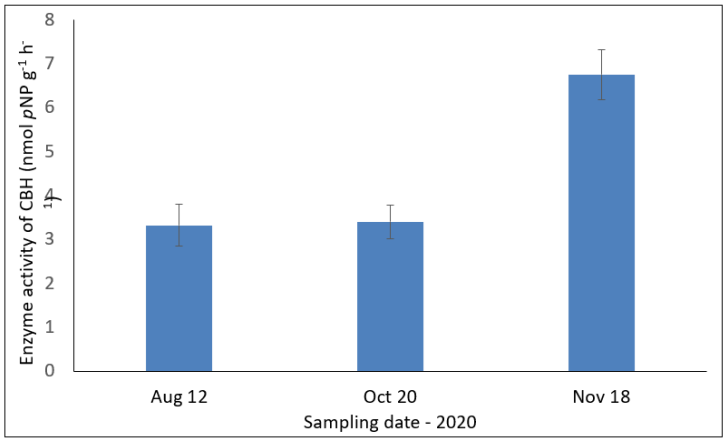

Fig. 3: Activities of Cellobiohydrolase (CBH), at different sampling dates. No significant difference was observed between means of CBH activities (nmol pNP g-1 h-1) for different consecutive sampling times (dates) based on the paired T-test (P≥0.05).

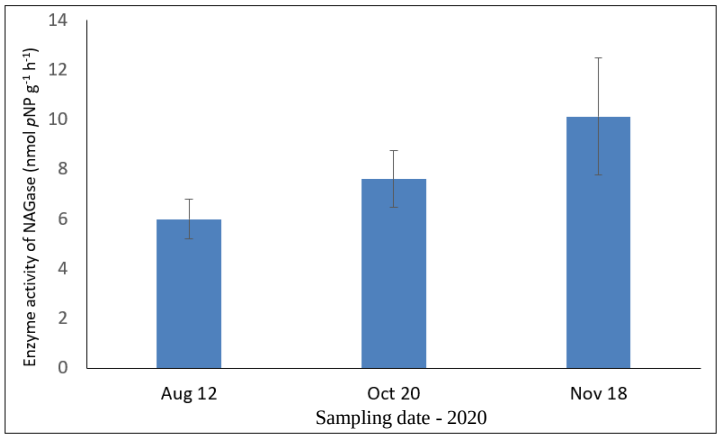

Fig. 4: Activities of β-N-acetylglucosaminidase (NAGase), at different sampling dates. No significant difference was observed between means of NA Gase activities (nmol pNP g-1 h-1) for different consecutive sampling times (dates) based on the paired T-test (P≥0.05)

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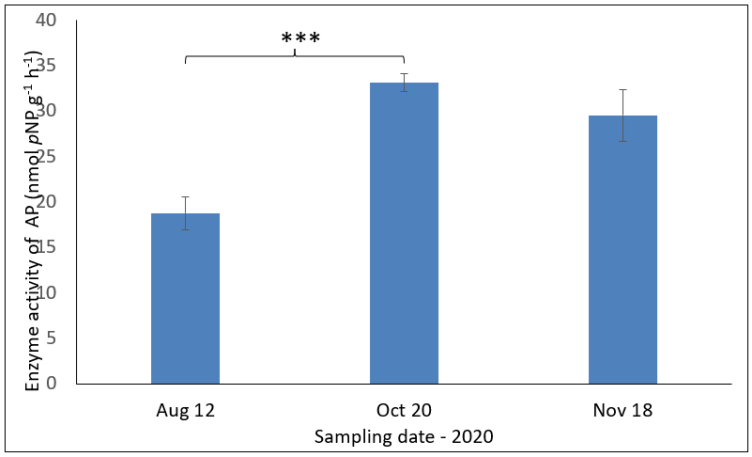

Fig. 5: Activities of Acid Phosphatase (AP), at different sampling dates. *, ** and *** represent significant differences between means of AP activities (nmol pNP g-1 h-1) for different consecutive sampling times (dates) based on the paired T-test (P≤0.05, P≤0.01, and P≤0.001 respectively)

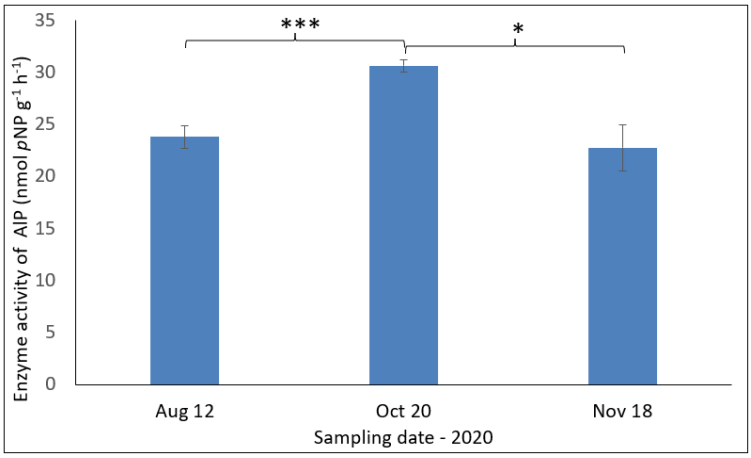

Fig. 6: Activities of AlP at different sampling dates. *, ** and *** represent significant differences between means of AIP activities (nmol pNP g-1 h-1) for different consecutive sampling times (dates) based on the paired T-test (P≤0.05, P≤0.01, and P≤0.001 respectively)

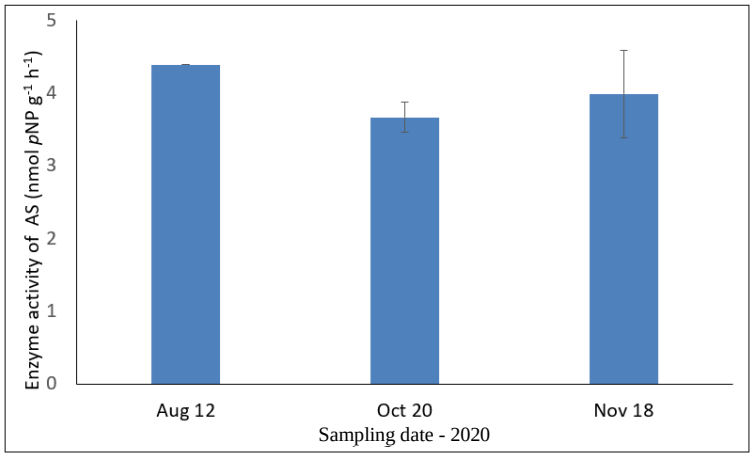

Fig. 7: Activities of Aryl Sulfatase (AS), at different sampling dates. No significant difference was observed between means of AS activities (nmol pNP g-1 h-1) for different consecutive sampling times (dates) based on the paired T-test (P≥0.05)

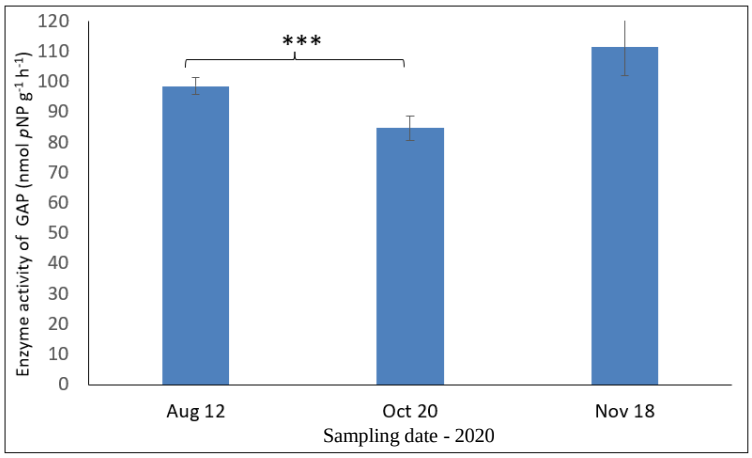

Fig. 8: Activities of GAP at different dates. *, ** and *** represent significant differences between means of GAP activities (nmol pNP g-1 h-1) for different sampling times (dates) based on the paired T-test (P≤0.05, P≤0.01, and P≤0.001 respectively)

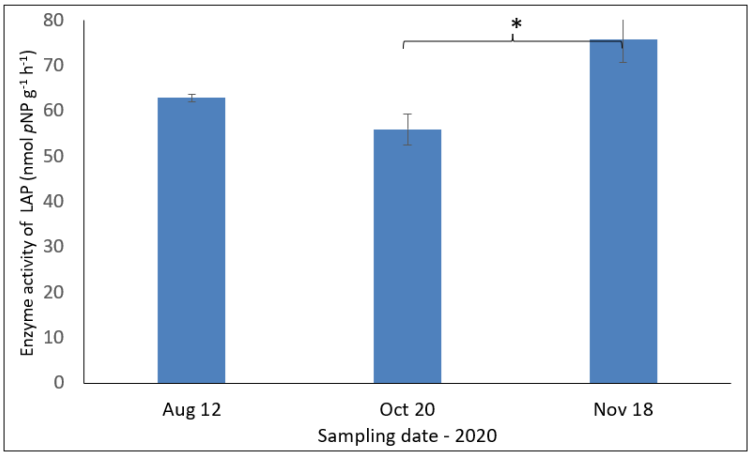

Fig. 9: Activities of LAP at different dates. *, ** and *** represent significant differences between means of LAP activities (nmol pNP g-1 h-1) for different sampling times (dates) based on the paired T-test (P≤0.05, P≤0.01, and P≤0.001 respectively)

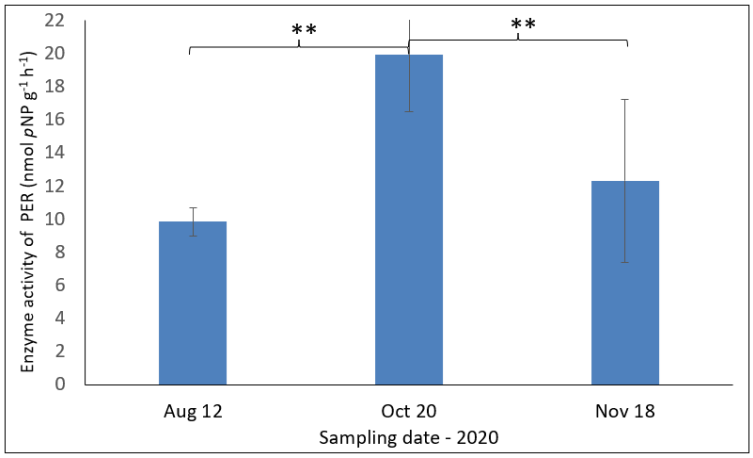

Fig. 10: Activities of PER at different dates. *, ** and *** represent significant differences between means of Peroxidase (PER) activities (nmol pNP g-1 h-1) for different sampling times (dates) based on the paired T-test (P≤0.05, P≤0.01, and P≤0.001 respectively)

Arylsulphatase Activity

AS activities were not affected by the seasonal variation of temperatures (Fig. 7). Studies done in different ecosystems reported an increase in AS activity, which did not support the findings (Castellano and Dick, 1991). AS positively benefits from higher C content in the soil and is driven more by substrate concentration and moisture as opposed to microbial biomass C or S (Fekete *et al*., 2011). Its broad substrate specificity also gives it an advantage in comparison to other enzymes when seasonal changes occur (Whalen and Warman, 1996).

Glycine Aminopeptidase and Leucine Aminopeptidase Activities

A significant decrease in GAP activity from August to October was expected, but the increase in LAP activity from October to November was a surprise (Fig 8 and 9). Studies showed that amino acid concentration was lowest in winter compared to other seasons (Di Filippo *et al.,* 2014). However, Norman *et al.,* (2020) demonstrated that aminopeptidases are unaffected by nitrogen availability, which would usually cause a more significant, proportional impact on other enzymes. Other studies observed a decrease in N availability and N mineralization at lower temperatures, but it is not known whether the aminopeptidases express this decrease and the results do not seem to support this (Turner and Henry, 2009).

Peroxidase (PER) Activity

The significant increase and decrease of PER activity across seasonal temperatures could have been driven by factors not related to temperature (Fig. 10). PER is more prevalent in fungi such as basidiomycetes and ascomycetes, therefore it is possible that an increase in fungi to bacterial ratio would allow existing fungi to use the resources and ATP from bacteria undergoing turnover (Zhang *et al.,* 2014, Abellan *et al.,* 2011). PER was previously characterized as having significantly higher variation among ecosystems in comparison to hydrolase activities. PER is also affected more by soil adsorption and yields by-products that strongly inhibit enzyme activity (Nicell and Wright, 1997). The instability in the organic matter makes the activity more susceptible to variations in temperature or substrate concentration in comparison to hydrolases (Sinsabaugh *et al*., 1991).

Conclusion

The temperature during the first sampling (August 12, 2020) was 21.3°C. It decreased significantly during the second sampling date (October 20, 2020) with a mean of 2.3°C and the third sampling (November 18, 2020) reaching -6.3°C. Enzyme activity remained unchanged for BG, CBH, NAGase, and AS whereas AP, AlP, GAP, LAP, and PER changed significantly overtimes. Other parameters such as microbial biomass and biodiversity, soil moisture, litter, C, N, S, and P might impact in situ enzyme response to decreasing temperatures. Data on enzymatic activities in soil samples from different ecological conditions should be interpreted with caution because of the various effects of environmental variations on soil functions.

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Author's Contributions

Alistar Moy: Experimented, analyzed the data, and write the first draft.

Kabwe Nkongolo: Designed the project, monitored data collection and analysis, and wrote the final version of the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues are involved.

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