***In vitro* sensitivity of forest soil enzymes to temperature increase in Western Patagonia**

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Abstract:

Soil enzymes can be sensitive indicators of C changes in forest soils produced by global warming. This study assessed the response to temperature of soil enzymes in forest soils from the fragile cold ecosystems of Western Patagonia (Chile), to be used as indicators of the biological C emissions from the soil under climate change for this Region. Soil from two sites of *Nothofagus* forest were sampled (0-5 cm), and soil C mineralization (21 days of soil incubation) and the enzyme activities of N-acetyl- β -glucosaminidase (NGA, linked to C and N cycles), β -glucosidase (BG, linked to C cycle), and endo-1,4- β -glucanase (CMCase, linked to C cycle) were measured at 5 °C and 20 °C. Additionally, sensitivity of enzyme activities was measured at 0, 5, 10 and 20 °C during experimental protocol incubation procedure, and energy activation (Ea) and Q₁₀ parameters were calculated. At both sites, the NGA activity mirrored the microbial activity, when temperature rose from 5 °C to 20 °C. The NGA showed Ea higher than BG, indicating that the release of N-acetyl-glucosamine was more sensitive to temperature than the release of glucose, related to BG. The results suggest the applicability of NGA as potential indicator to assess effects of global warming in forest soils from Patagonia.

Keywords: Activation energy, β -glucosidase, N-acetyl- β -glucosaminidase, C mineralization, global warming

1. Introduction

Soil enzymes have specific functions in the cycles of elements contained in the soil organic matter (SOM) and, have also been shown to be

sensitive indicators of environmental changes (Burns *et al.*, 2013). In forest ecosystems the soil enzymes can be sensitive indicators

of stress or changes produced by management activities, forest harvesting, or global increase of temperature by climate change (Baldrian *et al.*, 2013). The activity of soil enzymes can be influenced, among other factors by pH, moisture, substrate availability, and temperature. These factors can also affect soil microbial communities (size/composition), and therefore changes in these variables can affect overall enzyme production which subsequently alters the metabolic processes in the soils (Stursová and Baldrian, 2011; Adetunji *et al.* 2017). Particularly, the temperature is a factor that influences the enzyme catalysis rates, enzyme (or isoenzymes) production, and organic compound transformation rates in the ecosystems (Wallenstein *et al.*, 2011; Conant *et al.*, 2011). There is an increasing interest in determining the thermal sensitivity of certain enzymes that are considered as keys in the biogeochemical cycles of the principal elements in soils of different ecosystems, as a way to predict changes in the dynamics of the elements that could be triggered by global warming. The effect of temperature on the activity of soil enzymes is frequently estimated calculating the thermodynamic parameters, activation energy (E_a) and temperature coefficient (Q_{10}) from differential temperatures applied to the biochemical process. These parameters represent the temperature dependence of the enzyme reaction, providing a measurement of the sensitivity of the enzyme to warming conditions (Wallenstein *et al.*, 2011; Baldrian *et al.*, 2013; Bárta *et al.*, 2014; Blagodatskaya *et al.*, 2015; Razavi *et al.*, 2017). In forest soils, many organic polymeric substances are continually transformed by the action of microorganisms and their enzymes. The organic degradation is a combined (or synergistic) function of multienzyme complexes depolymerizing macromolecules to oligomers or monomers

(via extracellular enzymes). These, are easily assimilated by the microorganisms for its final metabolism (via intracellular enzymes), for example to CO_2 or NH_4^+ (Conant *et al.*, 2011). Such macromolecules are important due to their abundance and include the cellulose and the chitin, with some structural similarities, but also with great differences that are reflected in the enzymes that catalyze their decomposition. These are produced by different groups of microorganisms specialists, mainly fungi and bacteria (Baldrian and Valášková, 2008; Kellner and Vandenbol, 2010; Beier and Bertilsson, 2013). The evidence shows that in multistage reactions -such as cellulose and chitin degradation, each one of the different stages can display a different temperature dependence, which can individually affect the overall degradation process (Conant *et al.*, 2011). Therefore, the assessment of the activity of the enzymes involved in cellulose (exo- and endocellulases, and β -glucosidases) and chitin degradation (chitinases and N-acetyl- β -glucosaminidases), and their temperature dependence, is useful to understand how abiotic changes in soils from forest ecosystems can affect the cycle of C and N, i.e. effects of increase temperature due to global warming.

Climate change is affecting cold ecosystems of the Arctic, Antarctic and sub-Antarctic Regions. In the Patagonia Region it is anticipated that there will be an increase of up to 1.5 °C (IPCC, 2007). However, the uncertainty of the predictions is largely due to the lack of baseline data from those types of ecosystems. Nevertheless, previously observed changes of the C fluxes within different pools of SOM appear to be real, and it is thought that further characterization of soil enzyme activities may help to understand the net magnitude of SOM mineralization in response to increased temperatures.

The Western Patagonia Region, or the Chilean side, is a vast area that extends from latitudes 43°38'S to 56°30'S, along the west coast of South America, covering around 242,000 km². Climate conditions in the Region account for low temperatures and strong winds throughout the year, with very rainy weather conditions in the archipelago, and significantly lower precipitation on the Eastern Patagonian plains. The vegetation, strongly influenced by the extreme edaphoclimatic conditions, can vary from evergreen and deciduous forests, to peat lands and steppes (Flores *et al.*, 2010; Le Roux, 2012). The natural ecosystems of the Region are dominated by meadows that have developed on peat or Histosol soils under a tundra environment, and native forests of *Nothofagus* on soils that include Inceptisol and Spodosol Orders. Most of the forests are managed for commercial or conservation purposes, and the impact of climate change in this extended area has not been sufficiently studied in terms of the role of C (and N) in SOM under climate change scenarios. Considering that enzymes are an integral part of C and N cycling in SOM, it was hypothesized that they could be used to evaluate the dynamics and role of soil C in fragile cold ecosystems such as those from the sub-Antarctic Patagonia Region, in response to global increases of temperature. Therefore, the objective of this research was to determine the response of relevant soil enzymes involved in cellulose and chitin degradation, to the increment of temperature in two forest ecosystems from the Western Patagonia Region (Chile), and assess their potential to be used as indicators of increased SOM mineralization under climate change. Additionally, the investigation sought to determine the relationship between soil properties and enzyme activities within the SOM of the respective ecosystems.

2. Materials and Methods

2.1. Sites description

The study was conducted in forest soils from the Western Patagonia Region in South America. This is an extensive area dominated by relatively cold temperatures and low mean annual precipitation, and the native vegetation is composed of evergreen to deciduous forests, peat lands and steppes. In particular, the temperatures in the study area are from 1-4 °C in the cold season, which extends from March to November, and 10-20 °C during the warm season, from December to February (modelled data, in preparation for publication).

Two native forest sites were investigated, encompassing dominant deciduous species of *Nothofagus betuloides* at the Reserva Magallanes - RM forest (53°8'S, 71°0'W; 360 masl), and *Nothofagus pumilio* at Las Nieves - LN forest (52°17.34'S, 71°43.36'W; 551 masl). The soil at both sites were described and classified as Inceptisol, whereby the RM-forest soil was classified to the Family level as fine loamy, mixed, superactive, Aquandic Humicryepts, whilst the LN-forest soil was a coarse loamy, mixed, superactive, frigid, Spodic Dystrocryepts.

The sites differed somewhat in the amount of annual precipitation, specific soil properties, and the forest management. In particular the precipitation within the area of RM-forest is higher (600 mm year⁻¹) than in LN-forest area (300-500 mm year⁻¹). The soil properties of the respective sites are shown in Table 1. The level of SOM between the soils is similar, but there was higher total N at the RM-forest site. The site LN-forest showed the effect of the higher precipitation and coarser soil texture, which favours leaching of basic cations and nitrate in the soil solution, lower the soil pH and decreasing the soluble pool of N. The soil clay content is higher in the RM-forest site, and

consequently the water holding capacity was also higher on this site. The forest management at the RM-forest site included only shelterwood management

i) to maintain the existing biomass since the forest belongs to a National Park, and the LN-forest site had light commercial use ii) to extract wood products.

Table 1. Soil chemical and physical properties of two managed forest sites from Western Patagonia Region.

	Forest sites under study	
	Reserva Magallanes (RM-forest)	Las Nieves (LN-forest)
Soil chemical properties		
Soil pH ¹	5.96	5.51
SOM ² (%)	16.76	15.43
Soil physical properties		
Soil texture ³	Loam	Loamy-sand
Sand (g kg ⁻¹)	322	759
Silt (g kg ⁻¹)	482	190
Clay (g kg ⁻¹)	196	51
Bulk density ⁴ (ton m ⁻³)	0.69	0.64
Field capacity	62.5	36.3
Permanent wilting point	51.5	29.9

¹Soil: solution =1:5, determination in water extracts. ²Loss of ignition method. ³Pipette method. ⁴Cylinder method (0-5-cm depth

2.2. Soil sampling and processing

At each site, composite soil samples were collected from plots of 28 m² (n =4) which were randomly distributed over the area of 3 ha and 4 ha for RM-forest and LN-forest, respectively. The sampling was done at the beginning of the warm season at 0-5 cm depth of the mineral soil, to obtain the soil with the greatest microbial activity. Soil samples were taken at each site in one day, transported to the laboratory in cooling boxes and stored at 4°C. The soils were homogenized, sieved (2 mm) and sub-samples from each field replicate were adjusted to optimum moisture condition at 60%

of the water holding capacity (WHC). The WHC was calculated using measured water contents at the permanent wilting point (-1500 kPa), the field capacity point (-30 kPa), and the soil bulk density (Table 1). The actual gravimetric soil moisture was measured, and water was subsequently added or evaporated from the samples to reach 60% WHC. Additionally, part of each soil sample was not adjusted to the 0.6 WHC and was kept at field soil moisture in order to measure enzyme activities at standard conditions (field soil moisture and 37 °C). After adjusting the soil moisture contents, the soil samples were maintained at 4 °C during the two weeks before microbial and enzymatic assays.

2.3. Microbial C mineralization

The microbial activity was measured using soil incubation to determine C mineralization of SOM, as described by Alef and Nannipieri (1995). Soil samples of 25 g (x 3) were set into 1 L glass jars with a standardized 7 mL NaOH solution (0.5 M), reacting with the CO₂ resulting from the microbial decomposing activity during short time periods. Periodically, after 3, 7, 14, and 21 days of incubation the NaOH solution was removed and replaced from the jars to be subsequently titrated with HCl (0.1N) after addition 2 mL of BaCl₂ (1M). The 21-day period of soil incubation was selected as the final period for short term incubation because of the high content of organic matter in the soils and short periods of warmer temperatures where the samples were taken in the field. The procedure of soil incubation was repeated in a jar with no soil sample to measure the basal concentration of CO₂ in the jar, which was subtracted from the total evolved CO₂ from the other jars with soil samples. The C-CO₂ emitted from each period was summed to account for the total 21 days of assay, and the results were compared with the enzyme activities.

A soil incubation assay was conducted at the ideal temperature of 20 °C, and an additional one was established at 5 °C, in order to relate the response of the enzyme activities with the C decomposition according to the temperature. Thus, the enzyme activities measured under standard protocol conditions that are described below were compared with the microbial activity at standard condition of 20 °C, and then also at the lower temperature of 5 °C.

2.4. Enzyme activity

The standard activity of soil enzymes was measured at 37 °C, as is established in most protocols (Alef

and Nannipieri, 1995; Garcia *et al.*, 2003), and at field moisture soil content, which was expressed per gram of dry soil (gravimetric method). The activities of β -glucosidase (BG; EC 3.2.1.21) (Eivazi and Tabatabai, 1988; García *et al.*, 2003) related to cellulose degradation and N-acetyl- β -glucosaminidase (NGA; EC 3.2.1.52) (Parham and Deng, 2000) related to chitin degradation were determined on 1 g of soil using *p*-nitrophenyl- β -D-glucopyranoside and *p*-nitrophenyl-N-acetyl- β -D-glucosaminide, respectively, and the *p*-nitrophenol (*p*-NP) released after hydrolysis by incubation for 1 h was measured at 410 nm. The activity of both enzymes was quantified by reference to a calibration curve obtained with *p*-NP standards. Endo-1,4- β -glucanase or endocellulase activity (CMCase; EC 3.2.1.4) (Garcia *et al.*, 2003) also related to cellulose degradation was measured in 2.5 g of soil using carboxymethylcellulose (CMC) and the reducing sugars released from the substrate after 24 h of incubation were measured at 540 nm. The activity was expressed in terms of glucose. Control samples were prepared for each soil, adding the substrate after stopping the enzymatic reaction. The enzyme activities were expressed as μ mol of product released per gram of dry soil (oven-dried soil at 105 °C) and per hour of incubation (μ mol g⁻¹ soil h⁻¹). All determinations were performed in triplicate and the mean values are reported.

2.5. Temperature sensitivity assay

To assess the temperature dependence of enzymes within the range of local temperatures in the Region, the soil samples (60% WHC) were assayed modifying the temperature during the enzyme incubation protocol to 0, 5, 10 and 20 °C for the incubation period corresponding to each enzyme assay (1 h for BG and NAG and 24 h for CMCase). This type of experiment has been applied before by other authors

(Trasar-Cepeda *et al.*, 2007; Steinweg *et al.*, 2013; Bárta *et al.*, 2014), when they were evaluating the temperature dependence or thermal sensitivity of soil enzymes through the parameters activation energy (E_a) and/or temperature coefficient (Q_{10}). Due to a very low or no activity of endo-1,4- β -glucanase was detected at 5 °C, the measurement of activity for this enzyme was not carried out at 0 °C. The response of enzymes to temperature in the range 0-20 °C was evaluated calculating the parameters of E_a and Q_{10} . Arrhenius plots were constructed to estimate E_a ($\text{J K}^{-1}\text{mol}^{-1}$) according Arrhenius equation (Eq. 1):

$$k = Ae^{(-E_a/RT)} \quad \text{Equation 1}$$

where A, is the pre-exponential factor, E_a is the activation energy, R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$) and T is the absolute temperature in Kelvin (K). For each enzyme E_a was calculated from the slope of the plot obtained representing the natural log function of enzyme activities versus $1/T$. The Q_{10} values in the range 0-20 °C for each enzyme were calculated using equation (Eq. 2):

$$Q_{10} = e^{10E_a/RT(T+10)} \quad \text{Equation 2}$$

2.6. Statistical analysis

Linear regressions ($P \leq 0.05$) were applied to microbial and the enzyme activities to verify the association of the microbiological variables in the decomposition processes. Analyses of the variance were done (Genstat software 16th Ed.) in order to establish significant differences between the site enzyme activities and the temperature sensitivity parameters ($P \leq 0.05$). Least significant difference test (LSD) was used to separate the mean values of each assay ($P \leq 0.05$).

3. Results

3.1. Contrasted soil properties at the managed native forest sites

The sites were selected for the study because of the differences in the precipitation levels, where annual rainfall at RM-forest is higher than at LN-forest site, but the soil temperatures were within the same range. Nevertheless, there are differences between soil properties between the sites (0-5 cm depth) beyond the forest management, although both are taxonomically classified as Inceptisol soils (soil Order) with cold temperatures (Cryept Suborder). The main differences between soils can be summarized as follows: the soil at the RM-forest had less sand, more clay and was slightly less acidic than the soil at LN-forest; the soil at LN-forest was also found to have properties that intergrade to the Spodosol soil order, that would indicate a tendency for downward mobility and precipitation Fe and Al oxides (sesquioxides) in the soil profile.

The RM-forest soil has larger SOM content than LN-forest soil (Table 1) and therefore, larger total C and N contents as well, and total N was double the total N at the LN-forest soil (Table 2), as shown by the replicates measurement. The C:N ratio in RM-forest soil was also 6 units less than in LN-forest site. In terms of total mass of C within the top 5 cm of mineral soil, the C contained in the RM-forest soil was 40.8 ton ha^{-1} , considering the % C and bulk density indicated in Table 1, whilst the LN-forest soil had 8 ton ha^{-1} less than the other one. Using the equivalent information for the total N mass (Table 2), the difference between sites was $1.6 \text{ ton N ha}^{-1}$. The soil pH measured from soil samples at the pit also showed higher value at RM-forest soil, but the values measured in the soil samples used as replicates for microbial and enzymatic measurements (Table 2) showed no difference between soils ($P \leq 0.05$).

The physical properties between the sites differed mainly in soil texture and water holding capacity (Table 1). The clay proportion is higher in the RM-forest soil (10.5 % higher at RM-forest than LN-forest), and consequently the water holding capacity is higher in this site compared

to the LN-forest site. As with the pH values, the clay content variability was accounted by measuring at each replicate, and significant differences were found between soils ($P \leq 0.05$), where RM-forest soil showed higher clay content than LN-forest soil (Table 2).

Table 2. Soil properties measured at 0-5 cm depth at the replicate level (n=4) from two managed native forest soils from Western Patagonia Region.

	Reserva Magallanes (RM-forest)	Las Nieves (LN-forest)	F value	LSD value
Soil carbon ¹ (%)	11.84 ($\pm$ 3.92) ²	10.00 ($\pm$ 2.05)	0.433	ns
Soil nitrogen ¹ (%)	1.00 ($\pm$ 0.28)	0.56 ($\pm$ 0.10)	0.025**	0.36
Soil pH	5.47 ($\pm$ 0.38)	5.42 ($\pm$ 0.12)	0.809	ns
Soil clay content (%)	28.8 ($\pm$ 1.62)	21.4 ($\pm$ 3.68)	0.011**	4.96

¹Dry combustion method. ²Standard derivation in brackets. *no significant at $P < 0.05$. **significant value at $P < 0.05$

Table 3. Biochemical and biological properties of two managed native forest soils from Western Patagonia Region measured under standard conditions, respectively.

	Forest sites under study	
	Reserva Magallanes (RM-forest)	Las Nieves (LN-forest)
<u>Enzyme activity</u>		
β -glucosidase ¹ ($\mu\text{mol g}^{-1}\text{dry soil h}^{-1}$)	6.45 ($\pm$ 0.75) * b**	8.86 ($\pm$ 2.30) b
Endocellulase ¹ ($\mu\text{mol g}^{-1}\text{dry soil h}^{-1}$)	0.10 ($\pm$ 0.01) c	0.06 ($\pm$ 0.02) c
N-acetyl- β -glucosaminidase ¹ ($\mu\text{mol g}^{-1}\text{dry soil h}^{-1}$)	12.01 ($\pm$ 0.74) a	6.59 ($\pm$ 1.09) b
<u>Microbial activity</u>		
C mineralization ² ($\mu\text{g g}^{-1}\text{C-CO}_2\text{ 21 day}^{-1}$)	2370.30 ($\pm$ 309) a	1014.41 ($\pm$ 163) b

¹Enzyme activities were measured at 37 °C and soil field- moisture condition. ²C mineralization measured at 20 °C and 60% of water holding capacity *Standard deviation values in brackets (n=4) **Different letters represent significant differences in enzyme/ microbial activity between soils ($P < 0.05$)

3.2. Soil microbial activity

The C mineralization, resulted from microbial activity measured during 21 days of soil incubation under

standard conditions (60% WHC and 20 °C incubation temperature), showed significant differences between sites ($P \leq 0.05$). The microbial activity was higher under RM-forest soil, approximately doubled the

microbial mineralization under the LN-forest soil, as shown at the end of the accumulated C-CO₂ in Figure 1, as summarized in Table 3. Within the soil incubation period, the accumulated soil C mineralization evolution showed a similar pattern over time at the two sites, where the microbial activity systematically increased at each period of evaluation, and there were significant differences

of the C-CO₂ mineralized during the periods between the sites (Figure 1). The C mineralization model for both soils was fitted to a linear function and implied that there was a high potential for C mineralization within the measured period of 21 days, but the microbial activity at the RM-forest soil was around twice the one measured at LN-forest soil at each assessed periods (Figure 1).

Figure 1. Microbial activity as C-CO₂ evolution affected by the temperature, measured at two managed native forest sites from the Western Patagonia Region.

3.3. Soil enzyme activities

The biochemical properties of the two forests sites were similar regarding the enzymes linked to the C cycle, but different in the NGA activity, an enzyme linked to the C and N cycles (Table 3). Specifically, no significant differences were found ($P > 0.05$) between sites regarding to the activity of BG and CMCase, which participate in cellulose degradation and are related to the C-cycle. In contrast, the activity of N and C-cycle enzyme NGA, involved in chitin degradation, showed significant differences ($P \leq 0.05$) between forest soils, being almost double in RM-forest soil, compared to the LN-forest soil. On the other hand, the

levels of CMCase activity measured in the soils from both forests were lower than BG and NGA activities. A trend among C mineralization and soil enzyme activities was positive only for the C respiration (21 days) and the NGA activity (Figure 2), and the calculated correlation coefficient was $r = 0.87$ ($P \leq 0.05$), with a 72 % of the variance accounted by a linear regression model between these two variables. The microbial activity showed also to be correlated with the clay content ($r = 0.71$, $P \leq 0.05$), and to a lesser extent with CMCase ($r = 0.51$). In turn, the NGA activity was correlated with the clay content ($r = 0.73$, $P \leq 0.05$) and CMCase activity ($r = 0.50$). The BG activity showed no significant ($P \leq 0.05$) and negative correlations with the other variables.

Cmin: carbon mineralized ($\mu\text{g g}^{-1} \text{C-CO}_2 \text{ 21 day}^{-1}$).

BG: β -glucosidase activity ($\mu\text{mol g}^{-1} \text{dry soil h}^{-1}$)

NGA: N-acetyl- β -glucosaminidase activity ($\mu\text{mol g}^{-1} \text{dry soil h}^{-1}$)

CMCase: Endocellulase activity ($\mu\text{mol g}^{-1} \text{dry soil h}^{-1}$)

Figure 2. Relationship between microbial and enzyme activities measured at two managed native forest sites from the Western Patagonia Region. Cmin: carbon mineralized ($\mu\text{g g}^{-1} \text{C-CO}_2 \text{ 21 day}^{-1}$). BG: β -glucosidase activity, NGA: N-acetyl- β -glucosaminidase activity, CMCase: endocellulase activity. Enzyme activities expressed as $\mu\text{mol g}^{-1} \text{dry soil h}^{-1}$.

3.4. Soil enzyme reaction to temperature

The response of the enzyme activities to the temperature under the determination procedure was compared with the soil microbial C mineralization activity, when the temperature was raised from 5 °C to 20 °C (Figure 3). The results showed a significant response of microbial activity ($P \leq 0.05$), since the soils were nearly four and six times activated by the temperature at the RM-forest and the LN-forest, respectively, when the temperature increased by a factor of four. This trend was also observed in the

activity of NGA and CMCase, mainly at RM-forest, as the BG showed some increase but not enough to multiply its activity. Although at standard temperature (37 °C), significant differences were not observed between the C-cycle enzymes at RM- and LN-forest (Table 3), at lower temperatures (≤ 20 °C) differences were observed between both forests, with the BG activity higher at RM-forest (Figure 3).

Considering the whole range of temperatures studied, the activities of BG and NGA were affected by the incubation temperature, with increasing activity values registered at higher temperatures (Figure 4).

The CMCase activity was less responsive to the increase of temperature from 5 °C to 10 °C, showing no activity at the LN-forest and very low activity at the RM-forest (Figure 4). In general, the RM-forest soil showed a greater C (and N) mineralization than LN-forest soil, since the enzyme activities were greater in that site at each temperature assessment. At the lowest temperature studied (0 °C) there was high activity of BG and NGA, which increased by approximately two and four times, respectively, when the temperature was raised from 0 °C to 20 °C. The BG and NGA reactions followed the Arrhenius equation (Eq. 1) when increasing temperatures from 0 °C to 20 °C in the two forest soils studied, which

enabled the calculation of the Ea values ($\text{kJ K}^{-1} \text{mol}^{-1}$) (Table 4) from the Arrhenius plots. The RM-forest site had soil enzyme activities with higher Ea than the LN-forest soil; in other words, the Ea measured from the BG and NGA activities were 60% higher in the RM-forest soil (Table 4). The Q_{10} values showed a decrease with the increase of temperature from 0 °C to 20 °C (Table 4). The values of Q_{10} were higher for NGA ($Q_{10} = 1.8$, mean value) compared to BG ($Q_{10} = 1.4$, mean value), and the RM-forest showed the highest Q_{10} values of the two sites (Table 4), which followed the same pattern as for Ea values. The insufficient data for CMCase limited the determination of parameters Ea and Q_{10} using Arrhenius model.

Figure 3. Microbial activity reaction to the increase of temperature (5 and 20 °C), and the response of enzyme activity during the same increase of temperature under protocol procedure for the correspondent determination. Different letters represent significant differences in enzyme/microbial activity between forest soils ($P \leq 0.05$).

Figure 4. Response of soil enzyme activities to the increase of temperature measured in two managed native forest sites from the Western Patagonia Region.

Table 4. Activation energy (Ea) and Q₁₀ values for soil enzymes in two native managed forest sites in Western Patagonia Region.

	Ea (kJ K ⁻¹ mol ⁻¹)		Q ₁₀							
	BG	NGA	BG				NGA			
			0°C	5°C	10°C	20°C	0°C	5°C	10°C	20°C
RM forest	28.7 (± 2.3)* a**	47.7 (± 8.5) a	1.57	1.54	1.52	1.48	2.12	2.08	2.01	1.92
LN forest	18.0 (± 4.2) b	33.1 (± 2.2) b	1.33	1.31	1.30	1.28	1.67	1.64	1.62	1.56

BG:β-glucosidase; NAG: N-acetyl-β-glucosaminidase.*Standard derivation values in brackets (n=4)**Different letters represent significant differences in Ea between soils (P<0.005)

4. Discussion

4.1. Soil biological and biochemical function under native forests studied at Western Patagonia.

The native forest sites studied from the Patagonia Region have been under sustainable management as a recognized valuable natural resource for environmental, touristic, (RM-forest) and commercial use (LN-forest). The shelterwood management applied to the forests improves the light incidence through the canopy to the soil, as well as the air circulation, and promotes C mineralization in the soil because of the rise in temperature, at least in the top mineral soil, i.e., the soil layer studied here (0-5 cm). The forest soils sustain a large stock of C, and modifications in the moisture and temperature conditions can influence the feedback of CO₂ to the atmosphere through soil respiration (roots and heterotrophic respiration). Therefore, the global increase of temperature would have a direct incidence in the warming of this kind of ecosystems. There were differences in some of the soil properties used to describe the soils, but when they were analysed among replicates, the main differences (P≤ 0.05) between the soils were essentially the clay content and the organic N content. The clay content is determined by pedogenesis processes (not influenced

by forest management), and it is a variable linked to SOM, influencing the C mineralization by potentially protecting the C substrates from microbial attack, within the soil aggregates (Zimmermann *et al.*, 2012; Merino *et al.*, 2016). Thus, the soils from the two sites were recognized to be different and metabolic results from enzyme and microbial activities were therefore expected (Vinhai-Freitas *et al.*, 2017). In addition to the clay content, the differences of total N between sites can be associated to the more humid conditions predominant within the RM-forest site, where the annual precipitation is higher than in LN-forest site and the annual aboveground net primary productivity might also be greater at RM-forest. Soil microbial activity measured as C-CO₂ production showed an active and a linear response over time (Figure 1) which is explained by the large C content of forest soils in the Region (Alauzis *et al.*, 2004; Dubé *et al.*, 2009). The C-CO₂ respiration from RM-forest showed to be within the range of other native forest ecosystems from North Patagonia and cold alpine soils (Dubé *et al.*, 2009; Córdova *et al.*, 2012), but the C mineralization from LN-forest was comparatively lower. Perhaps the shelterwood management compensates the local low temperatures, through higher forest residues and light intensity of use over the soil, as the more intensive the applied management of ecosystems in Patagonia, the higher

the C decomposition activity (Peri *et al.*, 2015). The RM-forest showed greater microbial activity ($P < 0.05$) than LN-forest, and it was partly explained by the clay content and less acidic pH, demonstrated by the significant regression analysis ($P \leq 0.05$).

In terms of the enzymatic activities measured at both sites, only the NGA showed the same tendency as was observed for microbial activity, indicating the same magnitude of differences between sites, where the RM-forest soil showed twice the activity that was measured in the LN-forest. The NGA activity also was positively correlated with the clay content. The activities of BG, and NGA in these forest soils are within the range -or higher than reported in other studies (García *et al.*, 2003; Trasar-Cepeda *et al.*, 2008), and slightly higher than soils from previously investigated Antarctic ecosystems (Machuca *et al.*, 2015). This can be an indicator of a greater C and N decomposition activity in forest ecosystems of Patagonia compared to Antarctica ecosystem, and therefore, a signal of the applicability of the enzyme activities to study C, N transformation in the soil. The level of CMCase activity measured in these soils was very low compared with BG and NGA activities, but was similar with those described under similar assay conditions using carboxymethylcellulose as substrate, at 30 °C (Pancholy and Raice, 1973) and 50 °C (Trasar-Cepeda *et al.*, 2007, 2008). Although, in most studies the activity of CMCase is measured at 50 °C (optimum temperature range 50-70 °C) for this enzyme under laboratory conditions (Baldrian and Valášková, 2008), in this study the temperatures used were lower than 50 °C, since the focus was to obtain results that are pertinent to natural conditions of the cold ecosystems of Patagonia forest soils.

4.2. Temperature effect on soil microbiological activity and the potential contribution to the C-CO₂ emission.

The potential contribution to atmospheric CO₂ from heterotrophic respiration is reflected under standard incubation temperature, although this temperature occurs only occasionally in the Region during the short summers, it would however, be reached from time to time. As the temperature in the Region is usually < 20 °C, a low value of 5 °C was chosen to screen the microbiological response to the increase of temperature, because this is just above the limit of the kinetic activation of soil microorganisms (Alef and Nannipieri, 1995). The four fold increase of temperature was reflected in five to six magnitude increase of microbial C-CO₂ production at RM-forest and LN-forest soils, respectively, which in terms of climate change scenarios of global warming, demonstrates the fragility of these ecosystems.

The comparison between C respiration from microbial activity at increasing temperature (from 5 to 20 °C) and the enzyme activities (Figure 3) shows that the NGA followed the trend of the C respiration, which has been reported before (Tabatabai *et al.*, 2010). In the same way the correlation of NGA activity with fungal biomass in soils (Miller *et al.*, 1998), organic C and N, and microbial biomass have been shown (Tabatabai *et al.*, 2010), all of these parameters being key components of forest soils. The activity of NGA in Patagonia forests was more related to the microbial activity (C respiration), than BG and CMCase activities, probably because it catalyzes the release of residues of N-acetyl-β-D-glucosamine from chitin (in combined action with chitinases), contributing to the microbial C and N pools, and increasing the rate

of respiration (Kellner and Vandenbol, 2010; Beier and Bertilsson, 2013). N-acetyl- β -D-glucosamine residues are important in soils since some bacteria build their own cell walls of murein using the residues, whilst others use these residues for energy production in catabolic processes (Beier and Bertilsson, 2013). Transcripts encoding NGA have been identified from basidiomycetes and ascomycetes fungi in forest soils (Kellner and Vandenbol, 2010), and its relevance to N mobilization by ectomycorrhizal fungi for their own nutrition or for host tree nutrition have been described (Courty *et al.*, 2008). These results demonstrated the importance of NGA in the final step of process of chitin degradation in soils and how through the products of its catalytic action can contribute to the composition and size of soil microbial communities through of control of ratio C/N in soils.

At the time of soil sampling, abundant fungal mycelium was observed in the site, mainly in RM-forest soils, but was not quantified in this study. However, the observation of high amount of fungal biomass in the soil can be associated with the NGA activity measured in this site, likely to be produced by soil fungi. In this way, the supply of chitin in these soils could be provided by the fungal biomass (cell walls), stimulating the microbial activity. Russell (2014) demonstrated through a field trial that the chitin added to tropical forest soils was rapidly metabolized, leading to a large increase in the soil respiration, contrary to the cellulose, which was slowly metabolized. This could indicate that the role of chitin could be more important than cellulose, as a decay compound for microbial activity in RM-forest soil, explaining the link between C substrate, NGA, and microbial activity, and C-CO₂ as a by-product. Therefore, the results obtained in this study can be used to interpret the soil C emissions from biological activity and the enzyme NGA in forest soils of Patagonia, and further contribute to explain

the response of these forest soils to global warming due to climate change.

4.3. Enzyme responses to the temperature under experimental protocol procedure.

An additional experiment was set, in order to test the enzymes sensitivity under potential increase of temperature in Patagonia. This was done by modifying the protocol temperature during the incubation time of the BG and NGA enzyme activities (Eivazi and Tabatabai, 1988; Parham and Deng, 2000; García *et al.*, 2003), to the range of predominant temperatures in the Region. The values of Ea obtained under assays conducted with increasing temperatures, showed that BG and NGA enzymes from RM-forest site have greater Ea, implying that in this particular site the effect of temperature on biopolymer decomposition would be greater than in LN-forest site. The differences in Ea between both forest sites may be related to different soil properties affecting the enzymes, or the substrates availability (cellulose and chitin), the soil microbial community composition, or the production of different isoenzymes in each site. All of these, have potentially been described as factors affecting the temperature sensitivity (Ea) of soil enzymes (Wallenstein *et al.*, 2011; Zimmermann *et al.*, 2012; Bárta *et al.*, 2014). The sites were indeed different, as the RM-forest soil had higher C and N contents under more humid conditions, greater fungal mycelium biomass, resulting not only in higher rates of C respiration and enzyme activities over the temperature range studied, but also the higher thermal sensitivities (high values Ea and Q₁₀) of enzymes.

The Ea of the reaction catalysed by NGA was higher than that of BG, showing that the hydrolysis of (1 $\rightarrow$ 4)- β -glycoside bonds by NGA during the final step of chitin degradation was more sensitive to changes in temperature than that of BG catalyzing

the hydrolysis during the final step of cellulose decay. Thus, in the event of an increase of temperature, the release of N-acetyl- β -D-glucosamine residues (or release of C and N) by NGA would be stimulated and so the microbial activity in forest soils, particularly at RM-forest. In turn, under the same temperature condition the reaction catalyzed by BG would be a rate-limiting step of glucose release as a source of energy for microbial metabolism in soils, mainly in LN-forest where BG showed the lower sensitivity.

Although there are few studies describing the temperature dependence (E_a) of NGA in soils, the E_a values of this enzyme in Patagonia forest (40 kJ K⁻¹ mol⁻¹ mean value) were within the range described in the literature (25-58 kJ K⁻¹ mol⁻¹) (Parham and Deng, 2000; Blagodatskaya *et al.*, 2016). The reported E_a values for the BG enzyme activity (26-59 kJ K⁻¹ mol⁻¹) (Eivazi and Tabatabai, 1988; Trasar-Cepeda *et al.*, 2007; Steinweg *et al.*, 2013; Blagodatskaya *et al.*, 2016; Razavi *et al.*, 2017), are usually larger than those for NGA enzyme. However, the BG- E_a obtained in this study was low (23 kJ K⁻¹ mol⁻¹ mean value) compared with other studies, particularly for LN-forest (18 kJ K⁻¹ mol⁻¹). The low values of E_a found for BG at LN-forest could suggest an adaptation of this enzyme (or its microorganism producers) to catalyze reactions in the cold forest ecosystems which therefore would show lower temperature dependency (Wallenstein *et al.*, 2011; Razavi *et al.*, 2017). It is possible that CMCase displays a similar trend to BG in these soils, with the lower temperature dependence than NGA. However, it was not possible to determine the thermodynamic parameters in the range of studied temperatures, since the endocellulase presented very low or no activity at temperatures most common in the soils of the Patagonia forests (< 20 °C).

In general, it is assumed that the enzyme activity doubles when the temperature increases by 10 °C (i.e., $Q_{10} = 2$); however, data from the literature frequently

report Q_{10} values < 2 for soil enzymes (Parham and Deng, 2000; Trasar-Cepeda *et al.*, 2007; Bárta *et al.*, 2014). The Q_{10} values obtained in this study were greater than 2 (for 0 °C to 10 °C range) only for NGA activity at RM-forest, and the values decreased as temperature was increased, but the differences were not significant ($P > 0.05$).

The enzymes studied here (BG and NGA) are representative of the degradation processes of the major biopolymers in soils: cellulose and chitin. Thus, the differences found in temperature sensitivities (E_a NGA > E_a BG) can contribute to the understanding on how the effect of temperature can influence the rate of degradation and the flows of C and N in Patagonia forest soils. In particular, the fact that NGA was more sensitive to temperatures than BG implies a simultaneous effect on the cycles of C and N, with implications regarding microbial activity and the subsequent emissions of CO₂-C from the soil. The sensitivity of the enzymes to the temperature can be affected by a series of environmental factors (Wallenstein *et al.*, 2011), which are not considered under laboratory conditions. The observed trends in this investigation were clear and there were important measured differences among the enzymes that were subjected to identical assay conditions.

The results obtained here, can lead to further studies where C components of SOM, microbial activity and NGA, are related under short, medium, and long-term incubations at different temperatures, in order to better understand their role in soil C emission, and model the C-CO₂ emissions from the biochemical pools. Thus, a more accurate determination could be obtained by testing and monitoring the effect of climate change in forest ecosystems from cold environments. The biochemical parameters that are associated to the equation of Michaelis-Menten, maximal catalytic rate (V_{max}) and half saturation constant (K_m), could

also help to explain the mechanisms involved in the enzyme activities, such as the recently published work of Razavi *et al.* (2017).

Conclusion

The results from short soil incubations showed that CO₂ from forest soils could actively contribute to global warming, as demonstrated by the linear increasing evolution of the C-CO₂ response, which was dependent on the clay content. Among the enzymes tested in this study, the NGA activity linked to C and N-cycles was related to the soil conditions of the two forest sites, which were subjected to shelterwood management as a strategy to maintain productivity and preservation. The NGA activity was correlated with the clay content and the microbial C mineralization thereby showing its function in the release of CO₂ from soil to the atmosphere. In addition, this enzyme displayed a higher activation energy than BG and therefore had a higher sensitivity to temperature. Under the light of the results of this investigation, and considering the current state of the natural resources of the Western Patagonia Region, it seems plausible that NGA activity in soil can be used as an indicator of perturbations of ecosystems in response to increasing temperatures from climate change. This would be particularly beneficial in this Region of the world, where the predictions of future climatic trends are not so straightforward. Further studies should consider using short, medium and long-term soil incubations relating microbial and NGA activities to further elucidate to role of enzymes in Patagonia soils.

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