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Soil Nutrient Availability Regulates Microbial Community Composition and Enzymatic Activities at Different Soil Depths along an Elevation Gradient in the Nanling Nature Reserve, China

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Abstract: Improving our understanding of how soil microbial community composition and enzyme activities vary with elevation will elucidate the impact of climate change on ecosystem function. We collected soil samples at three elevations (1000 m, 1200 m, 1400 m) from two soil depths in a subtropical forest in the Nanling Nature Reserve to analyze soil nutrient availability and the Gram-positive (GP) to Gram-negative (GN) bacteria ratio. We conducted a vector analysis of soil enzymatic stoichiometry to examine the spatial distribution of soil microbial C, N, and P limitations. The soil C:N ratio decreased with increasing elevation. The GP:GN ratio and vector length (read-outs of relative C versus nutrient limitation) were the highest at 1400 m due to lower C availability. At an elevation of 1200 m, lower P availability was reflected in higher soil C:P and N:P ratios and lower GP:GN ratios, as lower P availability suppressed microbial C decomposition. Furthermore, the GP:GN ratio and vector length showed contrasting responses to variations in soil depth. The validation of enzyme vector analysis to capture the responses of microbial community composition to soil properties is dependent on environmental conditions and should be considered in the development of future soil organic C (SOC) dynamics models.

Keywords: elevation gradients; Gram-positive to Gram-negative bacteria ratio; enzymatic vector analysis; forest ecosystems

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1. Introduction

Microbes play an important role in regulating the terrestrial C budget through influencing the decomposition and incorporation of organic materials into the soil [1]. Microbial community composition changes according to function, which requires resources typically sourced from the environment via extracellular enzyme action, thereby impacting soil processes [2,3]. Soil nutrient availability shapes ecosystem structure and function and generally varies with elevation; therefore, elevation gradients have been the focus of investigations on the impact of climate change on natural ecosystems [4–6]. Topsoil profiles generally exhibit large resource and environmental gradients [7,8]. Conversely, C inputs and nutrient availability are generally lower in the subsoil and thus may be limiting factors for microbial community composition and enzyme activity [8]. Previous studies have shown that climate and soil properties strongly influence resource availability [9]; thus, changes in these factors alter microbial community composition and enzyme activities.

Numerous studies have suggested that soil organic C is the principal growth-limiting resource for soil microbial communities; thus, microbial community composition may vary

with soil C availability [10–12]. It has been proposed that Gram-negative (GN) bacteria use more plant-derived C sources that contain some labile C, whereas Gram-positive (GP) bacteria use more soil organic-matter-derived C sources, which are generally recalcitrant. Thus, shifts in the GP:GN ratio may be used to assess microbial C availability in soils [8,13]. For example, the GP:GN ratio has been shown to increase with decreasing soil C availability either as soil depth increases or as the availability of labile substrates decreases [14,15]. Furthermore, the availability of other soil nutrients plays an important role in regulating microbial community composition. Orwin et al. [16] indicated that the GP:GN ratio was positively correlated with cellulose decomposition rates and high soil nutrient content, as GP bacteria targeting recalcitrant C require relatively high levels of N availability to support the production of the extracellular and transport enzymes required to utilize this nutrient source [17]. Therefore, it is important to understand the impact of substrate availability across environmental gradients in natural ecosystems on the structure and function of soil microbial communities.

The response of the microbial community to changes in resource availability may involve shifts in related enzyme activities. For example, Hernández and Hobbie [18] showed that the addition of labile C (i.e., glucose) to the soil increased C-related enzyme activity, likely due to the increase in GN bacteria in response to greater C availability. Similarly, Fanin et al. [19] indicated that GN bacteria were strongly linked to C-related enzymes in a microcosm experiment. Uncertainty remains regarding the role of specific groups of decomposers in the production of certain enzymes, yet this determination may be central to improving our understanding of the role of microbial community structure in ecosystem functions [19]. Moreover, analyses of individual enzymes can reveal differences in the absolute levels of activity between samples but provide little information about the overall behavior or nutritional status of microbial communities [20]. Enzyme activity vector analysis based on enzyme stoichiometry provides a quantitative measure of the relative resource use limitation of soil microbial communities and may indicate soil microbial C limitation, in addition to overall nutrient limitation [20,21]. Specifically, vector length represents the increased enzyme production required to acquire C relative to that for other nutrients (indicating microbial C limitation), and vector angle represents the enzyme production required to acquire P relative to that for N (microbial nutrient limitation) [20,21].

Resource-driven changes in soil microbial community composition play important roles in mediating enzyme allocation and the decomposition of specific C substrates [22–24]; thus, studying microbial responses to environmental changes may reveal the mechanisms of nutrient cycling. Further research is required to determine how the relationships between soil microbial community composition and enzyme activities may vary with future climate change [25]. Therefore, the aim of this study was to evaluate the variation in soil microbial community composition and enzyme activities at different elevations as well as at different soil depths. To this end, we analyzed microbial community composition, soil nutrient availability, and enzymatic activity along an elevation gradient and at two different soil depths in the Nanling Nature Reserve, China. Our hypotheses were as follows: (1) soil C and nutrient availability increase with elevation and decrease with soil depth, as reflected in the element concentration and stoichiometry [4]; (2) the GP:GN ratio decreases with increasing elevation and increases with increasing soil depth, corresponding to soil resource availability [8]; and (3) similar to the pattern of the GP:GN ratio, the enzyme vector length decreases with increasing elevation and increases with increasing soil depth.

2. Materials and Methods

2.1. Site Description and Sample Collection

The study was conducted in the Nanling National Nature Reserve in Guangdong province, China (24°37′–24°57′ N, 112°30′–113°04′ E), which covers an area of 58,368.4 hm^2 and has a typical subtropical monsoon climate. The annual average precipitation during the study period was 2108.4 mm, with average temperatures of 26.2 °C in the hottest month (July) and 7.1 °C in the coldest month (January) [26]. Soil samples were collected in

May 2020 from nine broadleaf mixed forest plots (40 × 60 m^2) distributed on the northern slope of a mountain within the reserve, in an area that is rarely disturbed by humans. Three plots were designated for each of the three selected elevations in the gradient (1000 m, 1200 m, 1400 m). Five soil core samples (5 cm diameter) were obtained from the surface (0–10 cm, after litter removal) and subsurface layers (10–20 cm) in each plot. The soil samples were stored on ice during transport to the laboratory. Altogether, 15 replicates for each elevation and soil depth were collected. All samples were passed through a 2 mm sieve to remove roots and stones. The soil samples designated for the enzyme assay were stored at −20 °C for less than 2 weeks.

2.2. Analysis of Soil Properties

Soil water content (SWC) was calculated as the difference in sample weight before and after being oven-dried at 105 °C for at least 48 h until a constant weight was reached. Soil pH was measured using a PB-10 pH meter (Sartorius, Gottingen, Germany) with a soil to water ratio of 1:2.5. Total organic C (TOC) was analyzed using an elemental analyzer (TOC-VCPH Shimadzu Corp., Kyoto, Japan), and total N (TN) was measured using a flow injection autoanalyzer (FIA, Lachat Instruments, Milwaukee, Brookfield, WI, USA). The total P (TP) was measured following H_2SO_4-$HClO_4$ digestion using the molybdenum antimony colorimetric method [27]. The TOC, TN, and TP were measured using 0.005–0.015 g of dried soil.

2.3. Composition of Soil Microbial Community

We used phospholipid fatty acids (PLFAs) to characterize the microbial community composition used as indicators of microbial nutrient limitation. We extracted the phospholipids from 4 g of freeze-dried soil using a solution with a 1:2:0.8 ratio of chloroform to methanol to citric acid buffer. After elution, the extracted phospholipids were separated using methanol on a silica column. The soil PLFA extracts were identified using an Agilent 6850 gas chromatograph (Agilent, Santa Clara, CA, USA). Fatty acids were denoted according to the PLFA nomenclature established by Zelles [28]. The ratio between GP and GN bacteria was calculated through dividing the sum of the phospholipid fatty acids indicative of GP bacteria (i15:0, a15:0, i16:0, i17:0, and a17:0) by the sum of the fatty acids indicative of GN bacteria (cy17:0, cy19:0,16:1w7c, and 18:1w7c). The PLFA concentrations were expressed as nmol PLFA g^{-1} dry soil.

2.4. Enzyme Analysis

The potential activities of C-acquiring enzymes (β-1,4-glucosidase [BG]), N-acquiring enzymes (β-1,4-N-acetaminophen glucosidase [NAG]), and P-acquiring enzymes (acid phosphomonoesterase [AP]) were measured following the protocol described by Nannipieri et al. [29]. Briefly, BG, NAG, and AP were measured through adding the substrates 4-nitroph-enyl-β-D-glucopyranoside, p-nitrophenyl-N-acetyl-β-D-glucosaminidine, and p-nitrophenyl-phosphate tetrahydrate, respectively, which bind to the chromogen p-nitrophenol [30]. Then, the samples were incubated at 37 °C for 1 h (BG and NAG) and 0.5 h (AP).

2.5. Vector Analysis for Measurement of Resource Limitation

To measure the extent of soil microbial C and other nutrient limitations, we used vector analysis of soil enzymatic stoichiometry following the method proposed by Moorhead et al. [21]. Notably, vector analysis provided measures of potential and relative resource use limitations rather than actual resource use limitations for soil microorganisms. Two metrics were calculated: vector length to quantify the relative C versus N and P limitation, and vector angle to determine the relative P versus N limitation [19,20] as follows:

$$Vector\ Length = SQRT(x^2 + y^2)$$
$$Vector\ Angle = Degrees(A\ tan\ 2(x, y))$$

where x represents the relative activities of C- versus N- and P-acquiring enzymes (BG/(BG+AP)), and y represents the relative activities of C- versus N-acquiring enzymes (BG/(BG+NAG) [20].

2.6. Statistical Analysis

All data were log-transformed following normality and homoscedasticity testing. A one-way analysis of variance (ANOVA) test was used to evaluate the effects of elevation on soil chemical properties, microbial community composition, and enzymatic parameters at each soil depth. Subsequently, mean comparisons were performed using Tukey's honest significant difference (HSD) multiple comparisons test ($p < 0.05$) using R Software v.4.1.1 (R Core Team, Vienna, Austria, 2020). All data were tested for independence using Chi-square. A canonical correspondence analysis (CCA) was conducted using the "Vegan" R package to identify the most important factors that shaped soil microbial community composition, and a hierarchical partitioning analysis was conducted using the "rdacca.hp" R package to calculate the relative influence of each factor [31]. Furthermore, Spearman correlation analysis was conducted to determine the relationships between enzyme activities, vector lengths, and vector angles and soil nutrient ratios, GP bacteria, GN bacteria, and GP:GN ratios for each soil depth. All graphs were plotted using the "ggplot2" R package.

3. Results

3.1. Soil Chemical Properties and Nutrient Stoichiometry

Soil chemical properties and nutrient stoichiometry patterns along the elevation gradient were similar at the two soil depths (Table 1). The average soil pH was significantly higher at elevations of 1000 and 1200 m compared to that at 1400 m. In contrast, the average SWC, TOC, and TN concentrations were significantly higher at an elevation of 1400 m than at elevations of 1000 and 1200 m. Similarly, the average TP concentration was highest at an elevation of 1400 m and lowest at 1200 m. The soil C:N ratio also increased significantly with elevation. Both the C:P ratio and the N:P ratio were significantly higher at an elevation of 1200 m than at elevations of 1000 and 1400 m (Table 1). Regardless of elevation, the average SWC, TOC, TN, TP, and N:P ratio were significantly lower in the subsurface soil than in the surface soil, whereas the soil pH, C:N ratio, and C:P ratio did not change with soil depth (Table 1).

Table 1. Soil chemical properties at different soil depths and elevations.

Soil Depth (cm)	Elevation (m)	pH (in Water)	SWC ($g \cdot g^{-1}$)	TOC ($g \cdot kg^{-1}$)	TN ($g \cdot kg^{-1}$)	TP ($g \cdot kg^{-1}$)	C:N	C:P	N:P
Surface Soil (0–10)	1000	4.7a (0.10)	0.27b (0.01)	89.2b (8.34)	3.55b (0.42)	0.23b (0.02)	28.17a (2.33)	437.1b (62.50)	16.60b (2.35)
	1200	4.60a (0.08)	0.25b (0.02)	101.61b (12.44)	4.35b (0.64)	0.15c (0.02)	24.39b (1.00)	676.3a (54.94)	28.86a (2.07)
	1400	4.14b (0.10)	0.45a (0.03)	213.12a (38.73)	10.23a (1.62)	0.38a (0.03)	20.11b (0.55)	539.2b (66.44)	26.21a (2.70)
Subsurface Soil (10–20)	1000	4.90a (0.08)	0.24b (0.01)	58.25b (5.32)	1.88b (0.27)	0.17b (0.02)	43.11a (7.74)	371.9b (50.95)	11.52b (1.83)
	1200	4.66a (0.07)	0.22b (0.01)	86.72b (10.18)	3.47b (0.48)	0.14c (0.01)	28.11b (2.93)	653.7a (68.67)	25.11a (3.14)
	1400	4.38b (0.10)	0.34a (0.01)	94.57a (10.75)	4.60a (0.52)	0.25a (0.02)	20.91b (0.62)	377.4b (28.22)	18.14a (1.25)

Notes: Values represent the mean and SE (in parentheses) ($n = 15$). Different lowercase letters indicate significant ($p < 0.05$) differences among elevations at two soil depths in one-way analysis of variance (ANOVA) based on Tukey's honest significant difference (HSD) comparison procedure. SWC = soil water content, TOC = total organic carbon concentration, TN = total nitrogen concentration, TP = total phosphorus concentration.

3.2. Effect of Elevation and Soil Depth on the GP:GN Ratio

The ratio of GP to GN bacteria was significantly affected by soil depth and was significantly lower in the subsurface soil than in the surface soil (Table 2). The GP:GN ratio was significantly affected by elevation and soil depth (Table 2, Figure 1). The GP:GN ratio in the surface soil was the highest at an elevation of 1400 m and the lowest at 1200 m, while that in the subsurface soil was the highest at 1000 m and the lowest at 1200 m. The average GP:GN ratio at each elevation was higher in the subsurface soil than in the surface soil (Table 2, Figure 1).

Overall, the soil properties and corresponding ratios represented by the two axes explained 87.98% and 93.69% of the variation in the GP:GN ratio in the surface and subsurface soils, respectively (Figure 2a,c). In the surface soil, the concentration of GP bacteria was positively correlated with the soil N and P concentrations (Figure 2a). In particular, soil N concentration, soil P concentration, soil N:P ratio, and soil pH were the main contributors to the variation in the surface soil GP:GN ratio, with soil N concentration providing the largest contribution (Figure 2b). In the subsurface soil, the concentration of GN bacteria was positively correlated with the soil C:P and N:P ratios. In particular, the soil C:N, pH, N:P ratio, and C:P ratio were the main contributors to the variation in the subsurface soil GP:GN ratio, with the soil C:N ratio providing the largest contribution (Figure 2d).

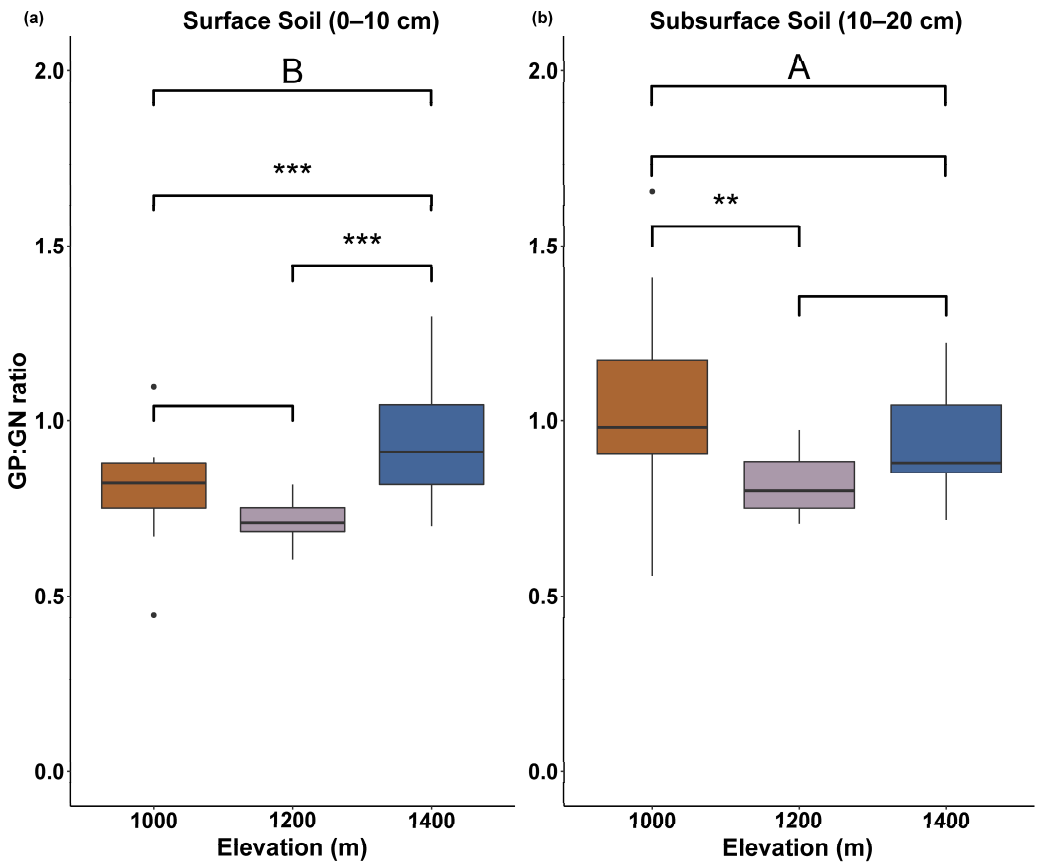

Figure 1. The effect of elevation on the ratio of Gram-positive (GP) and Gram-negative (GN) bacteria in surface soil (**a**) and subsurface soil (**b**). Boxes represent the central 50% of the data and the box whiskers represent 95% quantiles. ** indicates significant differences in GP:GN ratio among elevations; different capital letters indicate significant differences in GP:GN ratio between soil depths (Tukey's honest significant difference [HSD] test, $\alpha = 0.05$). ** indicates $p < 0.01$, *** indicates $p < 0.001$.

Table 2. Concentration of PLFAs as a biomarker of GP and GN bacteria and their ratio in soil profiles along the elevation gradient.

Soil Depth (cm)	Elevation (m)	GP (nmol g^{-1} Dry Soil)	GN (nmol g^{-1} Dry Soil)	GP:GN
Surface Soil (0–10)	1000	5.27a (0.34)	6.82a (0.56)	0.81ab (0.04)
	1200	5.47a (0.34)	7.71a (0.47)	0.72b (0.02)
	1400	5.37a (0.41)	6.04a (0.54)	0.91a (0.05)
Subsurface Soil (10–20)	1000	3.91a (0.38)	4.10a (0.51)	1.05a (0.07)
	1200	4.53a (0.27)	5.66a (0.43)	0.82b (0.02)
	1400	4.87a (0.33)	5.35a (0.47)	0.95ab (0.04)

Notes: Values represent the mean and SE (in parentheses) (n = 15). Different lowercase letters indicate significant ($p < 0.05$) differences among elevations at two soil depths in one-way analysis of variance (ANOVA) based on Tukey's honest significant difference (HSD) comparison procedure. PLFAs = phospholipid fatty acids, GP = Gram-positive, GN = Gram-negative.

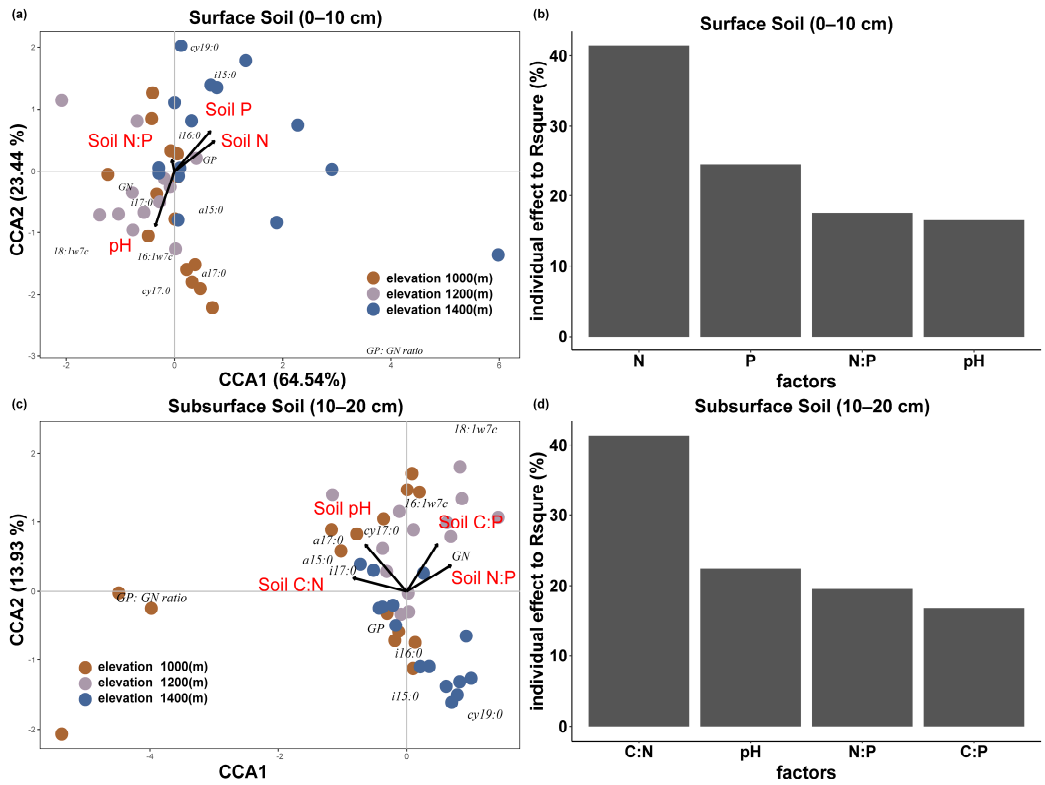

Figure 2. The canonical correspondence analysis (CCA) used to identify the relationships between microbial community composition and soil properties for surface soil (**a**) and subsurface soil (**c**). The GP:GN ratio is based on the phospholipid fatty acids (PLFA) analysis. The CCA analyses captured a large amount of variation in community composition in the first two components, with the two primary axes (CCA1 and CCA2) accounting for 87.98% (**a**, surface soil) and 93.69% (**c**, subsurface soil), respectively. Bar plots show the effect of individual factors on total R^2 at each soil depth (**b**,**d**).

3.3. Effect of Elevation and Soil Depth on Enzymatic Activities

Enzyme activity patterns varied across the elevation gradient at each soil depth (Table 3). Specifically, the BG activity in the surface soil increased significantly with increasing elevation, and the NAG and AP activities in the surface soil were higher at an

elevation of 1200 m than at 1000 and 1400 m. The vector length was longer at an elevation of 1400 m than at 1000 and 1200 m (Figure 3). Vector length was negatively associated with vector angle at an elevation of 1200 m at both soil depths (Figure 4).

Table 3. Variations in enzyme activity, vector length, and vector angle at different elevations and soil depths.

Soil Depth (cm)	Elevation (m)	BG (nmol g^{-1} Dry Soil)	NAG (nmol g^{-1} Dry Soil)	AP (nmol g^{-1} Dry Soil)	Vector Length	Vector Angle
Surface Soil (0–10)	1000	580b (62.5)	7.15ab (0.63)	22,064b (4839)	0.988b (0.001)	88.0a (0.22)
	1200	800ab (76.0)	11.7a (2.36)	36,150a (5761)	0.986b (0.002)	88.6a (0.11)
	1400	910a (79.9)	6.18b (0.43)	34,813ab (4894)	0.993a (0.001)	88.4a (0.14)
Subsurface Soil (10–20)	1000	317b (37.5)	4.93b (0.54)	11,954b (2515)	0.984b (0.002)	88.2a (0.19)
	1200	645a (110)	13.1a (3.11)	29,662a (6568)	0.982b (0.002)	88.6a (0.22)
	1400	469ab (48.1)	4.57b (0.50)	19,812a (3384)	0.990a (0.001)	88.5a (0.17)

Notes: Values represent the mean and SE (in parentheses) (n = 15). Different lowercase letters indicate significant ($p < 0.05$) differences among elevations at two soil depths in one-way analysis of variance (ANOVA) based on Tukey's honest significant difference (HSD) comparison procedure. BG = β-1,4-glucosidase, NAG = β-1,4-N-acetaminophen glucosidase, AP = acid phosphomonoesterase.

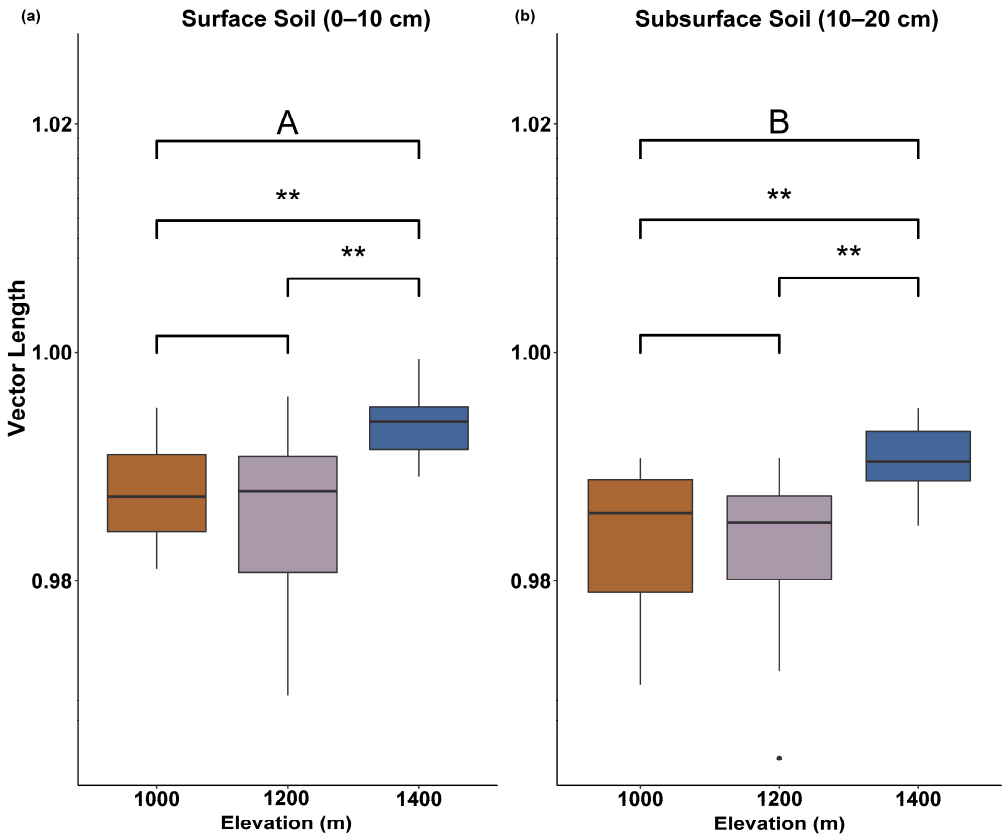

Figure 3. The effect of elevation on vector length (relative microbial C limitation) in surface soil (**a**) and subsurface soil (**b**). Boxes represent the central 50% of the data and box whiskers represent 95% quantiles. ** indicates significant differences in vector length along elevation gradient; different capital letters indicate significant differences in vector length between soil depths (Tukey's HSD test, $\alpha = 0.05$). ** indicates $p < 0.01$.

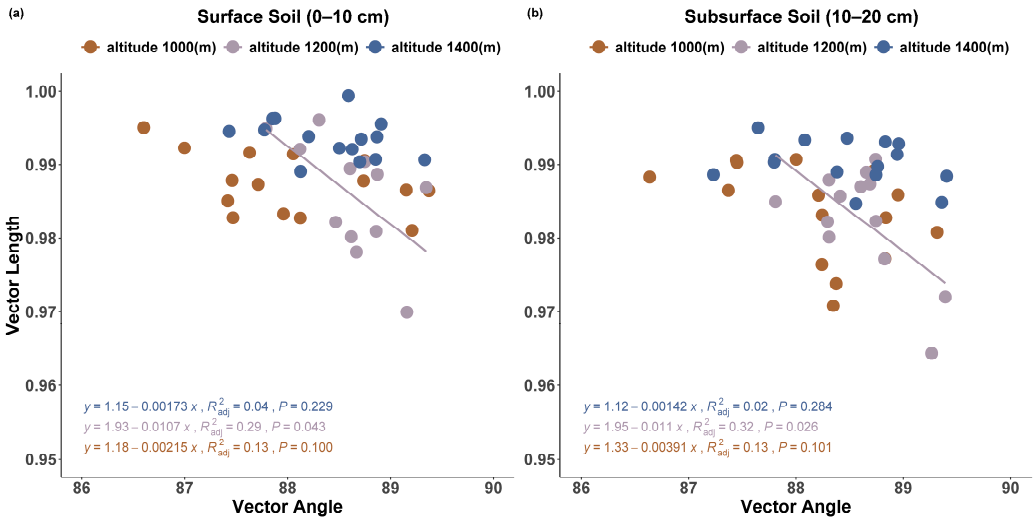

Figure 4. Relationship between vector length and vector angle along the elevation gradient in the surface soil (**a**) and subsurface soil (**b**). The y-axis denotes soil microbial C limitation, and the x-axis denotes soil microbial P vs. N limitation. Lines indicate a significant regression relationship (*p* values are provided in the figure above).

Overall, the activities of the three enzymes were positively correlated with the soil C:P and N:P ratios across soil depth. In the surface soil, BG activity was positively correlated with the amount of GP bacteria, while NAG activity and AP activity were positively related to the amount of GN bacteria and negatively related to the GP:GN ratio. In the subsurface soil, BG activity was positively correlated with the amount of GP and GN bacteria and negatively correlated with the GP:GN ratio. Similarly, NAG and AP activities were positively correlated with the amount of GN bacteria and negatively correlated with the GP:GN ratio. The vector length was negatively correlated with the soil C:N ratio across soil depth but only significantly positively correlated with the GP:GN ratio in the subsurface soil. The vector angle was positively related to the soil C:P and N:P ratios and negatively correlated with the GP:GN ratio across soil depth (Table 4).

Table 4. Spearman's correlation coefficients for soil nutrient stoichiometry, amounts of GP and GN bacteria, and GP:GN ratio with enzyme activities at different soil depths.

Soil Depth (cm)		C:N	C:P	N:P	GP	GN	GP:GN
Surface Soil (0–10)	BG	−0.26	0.56 ***	0.67 ***	0.39 *	0.25	0.02
	NAG	0.26	0.63 ***	0.61 ***	0.13	0.35 *	−0.55 ***
	AP	−0.06	0.80 ***	0.82 ***	0.27	0.37 *	−0.29
	Vector Length	−0.57 ***	−0.11	0.03	0.30	−0.03	0.41 **
	Vector Angle	−0.08	0.67 ***	0.63 ***	0.02	0.33 *	−0.44 **
Subsurface Soil (10–20)	BG	−0.30	0.66 ***	0.79 ***	0.47 **	0.65 ***	−0.74 ***
	NAG	0.09	0.77 ***	0.68 ***	0.17	0.44 ***	−0.72 ***
	AP	−0.07	0.83 ***	0.84 ***	0.24	0.47 ***	−0.70 ***
	Vector Length	−0.71 ***	−0.24	0.08	0.53 ***	0.33 *	0.00
	Vector Angle	0.25	0.55 ***	0.42 **	−0.18	0.00	−0.33 *

Notes: * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, $n = 45$. GP = Gram-positive, GN = Gram-negative, BG = β-1,4-glucosidase, NAG = β-1,4-N-acetaminophen glucosidase, AP = acid phosphomonoesterase.

4. Discussion

4.1. Soil Nutrient Availability Regulates GP:GN Ratio

Consistent with the results of previous studies [4,32–34], we found that soil organic C concentrations increased significantly with increasing elevation (Table 1), which may possibly be a result of low temperatures simultaneously constraining soil organic matter

turnover caused by the low temperatures that characterize high elevations [4]. Similar to the soil organic C concentration, the soil N concentration significantly increased with elevation, which may be explained by the close coupling typically exhibited between soil organic C and total N concentrations [4]. We determined that the soil C:N ratio decreased significantly as elevation increased, indicating increasing potential C limitation at higher elevations in the Nanling Nature Reserve, which does not support our first hypothesis. Correspondingly, the GP:GN ratio was highest at an elevation of 1400 m, which was contrary to our second hypothesis (Table 2).

The results of CCA demonstrated that soil N concentration and soil C:N ratio greatly influenced the variation in the GP:GN ratio in the surface and subsurface soil layers, respectively (Figure 2b,d). This influence may be due to an increase in the abundance of GP bacteria in response to increasing soil N availability and decreasing soil pH under higher N concentrations [14]. Additionally, GP bacteria have a higher tolerance for soil acidification than GN bacteria [35]. Furthermore, soil P availability was found to be one of the primary determinants of GP bacterial abundance, as GP bacteria require more nutrients as they target C resources [16,17]. This is reflected in the low GP:GN ratio at 1200 m of elevation, which is consistent with soil P availability patterns (Table 2). In particular, the lower P concentrations and higher soil C:P ratios at elevations of 1200 m provided less P for microorganisms [36].

The biomass and activity of microbes are generally constrained by the availability and quality of C and other nutrients [10,15,37]. The decline in soil C availability with increasing soil depth is predominantly a function of both decreasing C concentrations (Table 1) and reduced C quality as depth increases [9,37,38]. Correspondingly, the abundances of GP and GN bacteria were significantly lower in the subsurface soil than in the surface soil (Table 2). Furthermore, the results showed that the soil N:P ratio was significantly lower in the subsurface soil than in the surface soil, indicating higher relative soil P availability in the subsurface soil (Table 1). These results indicated that labile C input decreased and soil P availability increased in the subsurface soil [37–39].

4.2. Response of Enzyme Activities to Soil Nutrient Availability

Similar to the pattern of the GP:GN ratio, the vector length was negatively correlated with the soil C:N ratio (Table 4), as enzyme stoichiometry was sensitive to resource stoichiometry [24]. Furthermore, the results supported the supposition that microbial C limitation was not dominated by the total soil C content [9], likely because soil available C might be lower than the demand of soil microbial communities, which depends partially on microbial biomass [13]. According to previous studies, soil P limitation constrains microbial C metabolism through decreasing the decomposition rate of native soil C [40,41], which is reflected in the negative relationship between vector length and angle at an elevation of 1200 m (Figure 4). Microbes produce more enzymes targeting the increased abundance of substrates [42]; thus, specific enzyme activity decreased with soil depth, likely due to decreased C and other nutrient concentrations (Table 3).

4.3. Relationship between GP:GN Ratio and Enzyme Activities

Vector length was positively correlated with the GP:GN ratio in the surface soil (Table 4), supporting the use of the GP:GN ratio as an indicator of relative C availability [13]. However, no significant relationship was found between the GP:GN ratio and vector length in the subsurface soil (Table 4), likely because vector length is not an indicator of subsurface soil relative microbial C limitation [42,43]. Zheng et al. [42] indicated that enzyme stoichiometry indicates microbial resource limitations only when cellulose is the dominant C source. Microbes produce more N- and P-acquiring enzymes that require more C when lignin or lignin-embedded substrates and necromass dominate as microbial C sources, which induces contrasting enzyme stoichiometries [44].

As discussed previously, the decline in soil C and other nutrient concentrations with increasing soil depth reduced the abundance and activity of microbes, which was reflected

by the lower specific enzyme activity and abundance of bacteria in the subsurface soil compared to those in the surface soil (Table 3). However, the vector length was shorter in the subsurface soil, contrary to our third hypothesis and in contrast to the response of the GP:GN ratio to increasing soil depth. This result may be due to the invalidation of vector length as a function of microbial C limitation in the subsurface soil. Similarly, we found that NAG activity and vector angle did not change with increasing soil depth, likely due to the dual function of NAG as a C- and N-degrading enzyme [44,45]. In particular, a C shortage induces higher microbial production of NAG to allow microbes to obtain C from chitin, peptidoglycans, and proteins when more bio-available sources are scarce [44]. Thus, the validation of the enzymatic stoichiometry theory depends on soil C resources, in which microbes produce BG to target C when cellulose is the predominant C source (mainly in surface soil). However, microbes produce NAG to target C when chitin, peptidoglycan, and protein are the dominant C sources (mainly in deeper soil) [42,44]. Therefore, other N-acquiring enzymes, such as proteases and ureases, may provide a better indication of microbial N demand, depending on substrate availability.

5. Conclusions

Our study provides important insights into how soil substrate availability at different elevations and soil depths affects the GP:GN ratio and related enzyme activities. The results support the use of the GP:GN ratio as an indicator of soil C availability [13] and provide further support to recent claims that eco-enzymatic stoichiometry only reflects microbial resource limitations in certain contexts [43,46]. Owing to the one-time sampling in this experiment, we expect these results to be further validated under long-term experimental conditions. Other study limitations include only focusing on the soil chemical properties during one season and not evaluating the vegetation characteristics and soil physical properties of each sample site. Therefore, additional environmental factors should be included in future work. In addition, further studies are required to predict the patterns and couplings of microbial nutrient limitation at large spatial scales under global changes to better quantify the contributions of soil nutrient limitation to soil C dynamics. Future studies should incorporate PLFA analysis using more advanced techniques, such as next-generation sequencing, and more specific enzymes to provide an accurate and comprehensive picture of the dynamics of microbial community composition and their functions under environmental change.

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